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XXXIX. THE EXTRACTION OF PECTIN FROM THE FRUIT RIND OF THE LIME (*CITRUS MEDICA ACIDA*).

By FREDERICK HARDY.

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(*Received November 29th, 1923.*)

Introduction.

The extraction of pectin from plant tissues has recently been somewhat extensively investigated. In their work on the pectic substances contained in turnips, strawberries, rhubarb stem and apples, Schryver and Haynes [1916] employed a hot dilute solution of ammonium oxalate to extract pectinogen from the washed and dried material. Subsequently, Farnell [1923], working in conjunction with Schryver, showed that oxalic acid could be substituted for the salt, and indeed, is preferable in so far that it remains in solution when the extracted pectinogen is precipitated by alcohol, whereas ammonium oxalate is strongly adsorbed by the alcohol-gel. Farnell also demonstrated that solutions of ammonium sulphate and of carbonic acid are capable of extracting appreciable amounts of pectinogen from dried turnip, but that water alone is without effect. He suggested that pectinogen is loosely combined with calcium in plant tissues, and is liberated therefrom by any reagent which precipitates the metal. Carré [1922] has, however, shown that dilute hydrochloric acid is an effective extractor of pectinogen, a result which is not concordant with this generalisation.

In examining the factors which influence the extraction of pectinogen from the fibre of sugar cane, Farnell (private communication) has employed hydrochloric acid in preference to other reagents, and has demonstrated that the quantity of pectinogen extracted depends (*a*) on the degree of subdivision of the material, (*b*) on the length of time of extraction, and (*c*) on temperature. Maximum extraction was obtained in one to two hours; more prolonged treatment led to diminishing yield. Similarly, greater extraction was obtained at 98° than at 30°, but, when the material was extracted with acid in the autoclave at 120°, hydrolysis caused a marked reduction in the amount of pectinogen brought into solution. This last result was not observed, however, when acetic acid and when water were employed. Finally, lime water, of strong alkaline reaction, extracted no pectinogen from the fibre. Evidently the hydrogen ion concentration of the extracting agent is a factor of major importance in deciding the ultimate products of the reaction.

In order further to test this conclusion, and the more clearly to explain the results of other investigations, the writer has carried out a preliminary study of the behaviour of the pectic substance contained in the white inner rind of the fruit of the lime towards certain extracting agents.

Experimental.

The outer yellow rind of ripe lime fruits was removed with a sharp knife, and the inner white rind stripped off without rupturing the juice-containing pulp cells. The white rind was then minced in a machine, treated three or four times with alcohol to remove water, and finally twice extracted under a reflux condenser with boiling 95 % alcohol. The resulting material was air dried, passed through a sieve having circular holes of 2 mm. diameter, and bottled for future examination.

The following figures indicate the yield of rind residues obtained: 180 ripe lime fruits, of mass 9000 g. gave 288 g. of fresh white rind, which gave 100 g. of air-dry material, containing 9·3 g. of moisture, and 3·8 g. of ash (mainly calcium oxide).

The extracting agents employed in the investigation were water, dilute hydrochloric acid ($N/20$), citric acid solutions, and solutions of sodium hydroxide. The proportion of material to extracting solution was in every case 1 g. of material to 50 cc. of solution.

The amounts of pectinogen extracted are expressed in the accompanying tables as percentages (on the oven-dry basis) of calcium pectate, precipitated from measured volumes of the extracts by the procedure due to Haynes and Carré [1922]. For comparison, the percentages of alcohol-precipitable matter, and of total solids in the extracts were also determined. Alcohol precipitates were obtained by slowly delivering from a pipette small volumes of the extracts into ten times their volume of 95 % alcohol. Total solids were determined by evaporating known volumes of the extracts to constant mass, and correcting, where necessary, the mass of the residue for the amount of solid extracting agent present.

The well-known objections to the alcohol precipitation method of estimating pectic substances, and the errors involved in the method used for determining total solids, render the values obtained for these quantities unreliable. Hence trustworthy conclusions as to the relative effects of the various treatments described should mainly be drawn from the figures for calcium pectate precipitates, which are believed to be quantitative.

Viscosities were determined by observing the time of delivery of the extracts from a standard 50 cc. tubular pipette, and comparing the results with the time of delivery of water under constant temperature conditions. Reactions (p_H values) were estimated by the colorimetric method. In every case, the figures refer to the reaction of the extract and not to that of the extracting agent.

Table I. *Extraction of pectinogen from white lime rind with water.*

Treatment	Calcium pectate	Alcohol ppt.	Viscosity	Total solids	Ash in solids	p_H
1. Material allowed to remain in contact with water at 30° for 19 hours	18·1	22·7	1·093	27·3	1·2	5·2
2. Material put into water at 98°, and maintained at this temp. for 20 mins. ...	13·6	17·2	1·094	24·9	1·7	4·7
3. Material put into water at 98°, temp. then raised to 126° in autoclave and maintained for 20 mins.	33·5	45·9	1·031	59·4	1·4	4·8

Table II. *Extraction of pectinogen from white lime rind with dilute hydrochloric acid.*

Treatment	Calcium pectate	Alcohol ppt.	Viscosity	Total solids	Ash in solids	p_H
1. Material allowed to remain in contact with *N*/20 HCl at 30° for 19 hours ...	30·1	33·2	1·242	36·1	4·2	1·3
2. Ditto; temp. then raised to B.P. and maintained for 1 min.	35·9	43·5	1·355	57·0	4·6	1·3
3. Ditto; temp. then raised to 98°, and maintained for 1 hour on boiling water-bath (Carré method)	47·7	50·1	1·242	75·1	3·9	1·3
4. Material put into boiling *N*/20 HCl; temp. then raised to 126° in autoclave, and maintained for 1 hour	11·5	26·2	1·000	86·5	4·0	1·3
5. Material successively extracted by Carré method (see treatment 3):						
1st extract	48·2	51·7	1·182	78·3	2·8	1·3
2nd „	22·7	29·9	—	34·0	1·7	—
3rd „	11·5	6·5	—	18·0	0·6	—
4th „	5·0	3·2	—	7·2	0·4	—
10th „	0·1	—	—	—	—	—

Table III. *Extraction of pectinogen from white lime rind with solutions of citric acid.*

Treatment: (*a*) Material put into the acid solution at 98°; maintained at this temp. for 20 mins.; then allowed to cool for 10 mins.

(*b*) Material put into the acid solution at 98°; temp. then raised to 126° in an autoclave (10 mins.); maintained at this temp. for 20 mins.; then allowed to cool for 10 mins.

	Calcium pectate	Alcohol ppt.	Viscosity	Total solids	Ash in solids	p_H
(For comparison) *N*/20 HCl (Carré) ...	47·7	—	—	—	—	1·3
1·5 *N* citric acid 98°	42·8	52·8	1·182	—	—	2·0
126°	37·3	37·2	1·030	—	—	2·0
N/2 citric acid 98°	37·7	75·3	1·394	53·1	8·5	2·2
126°	41·8	55·1	1·091	61·8	6·2	2·2
N/20 citric acid 98°	18·6	25·7	1·090	34·4	8·6	3·2
126°	34·8	47·2	1·054	61·1	2·1	3·2
N/500 citric acid 98°	8·7	14·4	1·094	28·9	2·6	4·7
126°	30·6	35·4	1·063	57·3	2·0	4·8
(For comparison) water 98°	13·6	17·2	1·094	24·9	1·7	4·7
126°	33·5	45·9	1·031	59·4	1·4	4·8

Treatment: (*c*) Material allowed to remain in contact with *N*/2 citric acid at 30° for 19 hours; temp. then raised to boiling point, and maintained for 1 min.

(i)	48·3	56·2	—	—	—	2·2
(ii)	35·6	41·8	1·402	—	—	2·2

Table IV. *Extraction of pectinogen from white lime rind with solutions of sodium hydroxide.*

Treatment:—As with citric acid solutions (Table III)

		Calcium pectate	Alcohol ppt.	Viscosity	Total solids	Ash in solids	p_H
N/75 NaOH	98° ...	29·8	46·2	1·121	—	—	6·2
	126° ...	31·2	47·9	1·152	—	—	5·0
N/50 NaOH	98° ...	25·3	44·4	1·020	—	—	6·9
	126° ...	—	—	—	—	—	—
N/10 NaOH	98° ...	none	28·7	1·000	—	—	brown, >7·0
	126° ...	none	26·4	1·000	—	—	brown, >7·0
N/2 NaOH	98° ...	none	67·6	1·000	—	—	brown, >7·0
	126° ...	—	—	—	—	—	—

Table V. *Extraction of pectinogen from white lime rind under reduced pressure.*

Treatment: Material allowed to remain in contact with the extracting solution at 30° for 18 hours, then heated under reduced pressure (50 mm.) for 30 mins.

Extracting agent	Temp.	Calcium pectate	Viscosity	Reaction p_H
Water	50°	19·8	1·065	5·5
N/20 HCl	63°	27·6	1·350	1·3
N/2 citric acid	65°	28·6	1·402	2·2

Conclusions and Discussion.

The following conclusions are drawn from the data contained in the tables.

I. *Extraction with water.*

1. Water extracts considerable amounts of pectinogen from white lime rind. In this respect, the pectic substance of lime rind appears to differ somewhat from that contained in turnip, etc. which is insoluble in water. The reaction of the water extracts in the case of lime rind was distinctly acidic; this may account, at least in part, for the result observed.

2. Length of time of contact with water, and degree of temperature are factors that also affect the amount of pectinogen extracted.

3. The three factors, reaction, time, and temperature, apparently do not act altogether in the same direction. The data in Table I are not sufficient, however, to warrant definite conclusions as to the relative effects of these factors.

II. *Extraction with dilute hydrochloric acid.*

1. Dilute hydrochloric acid, at temperatures up to boiling point, extracts considerably more pectinogen from white lime rind than does water. [See also Report of Food Investigation Board, 1920, p. 24.]

2. Maximum extraction was obtained by the Carré method of treatment, but a single extraction is not sufficient to remove all the pectinogen from the material. Even after ten successive extractions with hot dilute hydrochloric acid, an appreciable amount of pectinogen, precipitable as calcium pectate, remained in the rind (Exp. 5, Table II). The substance remaining after extraction was partly soluble in cuprammonia and in a solution of zinc chloride;

it therefore presumably contained cellulose in addition to a resistant fibrous residue, probably of ligno-cellulose type.

3. Heating with dilute hydrochloric acid to high temperature in the autoclave brings about pronounced destruction of pectinogen in white lime rind. This result is similar to that observed by Farnell (private communication) for the pectic substance of sugar cane fibre.

III. *Extraction with solutions of citric acid.*

The series of results recorded in Table III for the amounts of pectinogen (calcium pectate) extracted by solutions of citric acid of different hydrogen ion concentration indicate the following relationships:

1. At 98° the amount of pectinogen extracted varies directly with the H ion concentration.

2. Destruction of pectinogen appears to be brought about at the higher H ion concentrations (p_H 2·0) by heating in the autoclave to 126°.

3. On the other hand, more pectinogen is extracted by the autoclave treatment when the H ion concentration is less than that represented by p_H 2·0 than by treatment at a lower temperature in open vessels. The difference is progressively less, however, the more acidic the solution.

IV. *Extraction with solutions of sodium hydroxide.*

1. Because of the acid reaction of solutions of pectinogen in water, treatment of lime rind with very dilute soda may yield extracts containing considerable quantities of pectinogen, provided the acidity is not neutralised by the alkali added.

2. When the final reaction is alkaline, a red-brown colour[1] develops, and the extract contains no pectinogen precipitable as calcium pectate. This result is in agreement with the observation of Clayson, Norris and Schryver [1921] that normal soda extracts no pectinogen from dried plant tissues, but a substance that is probably allied to the hemicelluloses, and with that of Farnell, that soda of medium dilution fails to extract pectinogen from sugar cane fibre (private communication).

V. *Relationship between alcohol precipitate, total solids and calcium pectate.*

The amount of alcohol-precipitable matter contained in the extracts is considerably greater than the amount of calcium pectate obtained, except in those extracts that possess low pectinogen contents, in which case, alcohol fails to precipitate the pectinogen completely. Haynes and Carré [1922] had previously recorded a similar observation.

[1] The alkaline extracts were similar in colour and odour to solutions obtained when glucose and certain gums, notably gum tragacanth, are heated with caustic soda. The colour may be due to the caramelisation of sugars produced by the hydrolysis of hemicelluloses, pentosans, etc. contained in the citrus rind, although a sample of rind after repeated extraction with $N/20$ HCl to remove pectinogen, gave a much less intense coloration than did unextracted rind. The colour is partially discharged by acidifying.

The total solid matter exceeds in amount both the calcium pectate and the alcohol precipitate, the difference being especially great in extracts obtained by autoclave treatment.

The above facts may be explained as due to the properties of the products of decomposition and of hydrolysis of the pectic substance occurring in white lime rind, and to the different amounts of ash components extracted by the various treatments.

VI. *Relationship between viscosity and pectinogen content.*

Although the viscosity measurements recorded in the tables are merely relative, they indicate that the physical structure of the colloidal matter present in the extracts is decided largely by the conditions under which the extracts were prepared. Thus, in every case where an extract was obtained by treatment in the autoclave, its viscosity was measurably lower than that of extracts obtained by other means, even though its pectinogen content were greater. Furthermore, extracts prepared under identical conditions of time and temperature, but in the presence of different concentrations of hydrogen ion, exhibited viscosities that appear to be functions of the degree of acidity rather than of pectinogen content. The correlation between viscosity and hydrogen ion concentration, however, is apparently not simple; the figures suggest an optimum p_H value at which the extracts exhibit maximum viscosity for constant pectinogen content. Finally, the time factor, with which should be associated the extent of the surface of contact between the pectinogen-containing material and the extracting solution, appears also to be a factor controlling, not only the quantity of pectinogen extracted, but also the viscosity of the extract obtained.

The complex circumstances that affect the viscosities of colloidal solutions have recently received considerable attention from investigators who have attempted to explain the properties of gels and sols in terms of physical structure. The now widely accepted micellar theory evolved by these investigators postulates the presence in colloidal solutions of primary particles of solvated matter which may possess various sizes and shapes, and which may become progressively aggregated to form granules or chains, fibrils or filaments, depending on the prevailing conditions. According to this theory, viscosity is believed to be the expression of the extent to which solvation and aggregation have progressed, and gelation, the final stage of the process. The fact that the viscosity of a colloidal solution depends not only on its concentration, but also on its previous treatment and history, is readily explained on these assumptions.

VII. *Possible relationship between viscosity and jellying power.*

Although the preliminary investigation described has not included direct determinations of the jellying power of pectinogen extracts prepared under different conditions, it seems reasonable to suppose that this property is

positively correlated with viscosity, since, in terms of the micellar theory, viscosity is a measure of the degree of incipient gelation. Hence, the values given for viscosities might be taken to indicate the jellying powers of the various extracts. Considered in this light, the data contained in the tables fall into line with certain experimental results of other investigators. Thus, in their researches on the jellying power of citrus pectin extracts, Johnstin and Denton [1923] noted that extracts possessing high jellying powers exhibited greater viscosities than extracts with less pronounced jelly-forming properties. They obtained no definite correlation, however, between the quantity of alcohol-precipitable matter and jellying power, and therefore concluded that the alcohol precipitation method is not a reliable means of estimating "effective pectin." Recently, H. A. Noyes [1923], in reviewing the efficacy of different processes for manufacturing jams and jellies, has stated that the best results are obtained by minimum heating, especially when carried out *in vacuo*, and that heating in open vessels destroys pectin, at least in so far as jelly producing power is concerned. The "vacuum cook process" is stated to be superior even to the "one minute cook process." In order to test this conclusion, the writer has submitted white lime rind to a vacuum extraction process with water, dilute hydrochloric acid and dilute citric acid solution. The results are set out in Table V. On comparing them with results obtained by other treatments, it is seen that the vacuum extracts possess the higher viscosities, considering their relatively lower pectinogen contents, and that, of the other treatments described, the one minute cook process is decidedly superior. These facts are in accordance with Noyes' experience.

It ought to be possible to replace by more exact means of procedure the empirical methods for preparing pectic products at present in vogue, by careful control of the complex factors involved in the processes of pectinogen extraction and jelly formation, and by the employment of precise quantitative methods for following the course of these processses. The application of the Haynes-Carré method of estimating pectinogen, and the use of the viscometer for comparing the colloidal conditions of pectic extracts should aid materially in bringing about this result.

Summary.

1. The primary object of the investigation was to examine the relative efficacy of water, hydrochloric acid, citric acid and caustic soda solutions of varying hydrogen ion concentration, in extracting, under different heat treatments, pectinogen from dried white rind of lime fruits.

2. Under similar time conditions, *the total quantity of pectinogen* extracted appears to vary directly with H ion concentration when extraction is carried out at temperatures below boiling point, and directly with temperature when the final reaction of the extract is less acidic than p_H 2·0.

Alkaline extracts possess a brown colour, and contain no pectinogen.

Autoclave treatment (126°) at concentrations of H ion greater than that represented by p_H 2·0, brings about destruction of pectinogen by hydrolysis.

3. The *viscosity* of the extracts appears to depend largely on the conditions of extraction rather than directly on pectinogen content. If viscosity be considered as a measure of the degree of incipient gelation, this observation is concordant with the experience of jelly manufacturers who have found that the jellying power of pectinogen solutions depends on the treatment to which the raw material is subjected.

The writer wishes to acknowledge his indebtedness to R. G. W. Farnell, Esq., Chemist to the British Empire Sugar Research Association, at whose suggestion the investigation was undertaken.

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XL. THE ERROR DUE TO IMPURE PICRIC ACID IN CREATININE ESTIMATIONS.

By CLIVE NEWCOMB.

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In the estimation of creatinine in very small concentrations, such as are met with in blood filtrates, by comparing the colours produced on the addition of alkali to the solution to be estimated and to a solution of known creatinine content, both saturated with picric acid, the purity of the picric acid used is an important factor. As picric acid can never be obtained without a perceptible amount of impurity, and is liable to deteriorate on keeping, it was considered that it might be better to work with picric acid admittedly impure and to estimate and allow for the impurity. No mention of the correction for the picric acid described in this paper having been found in a fairly careful search through the literature of the estimation, it was thought good to publish it, as, if it is found sound, it avoids any special purification of the picric acid, and either a great multiplication of unstable standards on the one hand, or lengthy calibrations of a few of them on the other.

In rough estimations of creatinine an assumption commonly made is, that the concentrations of the unknown and the standard are inversely proportional to the lengths of the columns in the colorimeter required for a match. That is to say, if

s is the concentration of the standard solution;
u ,, ,, unknown ,, ;
r is the reading of the colorimeter with the standard set at 10;

that

$$s/u = r/10 \text{ and hence } u = 10s/r.$$

Unless, however, the standard and the unknown are of nearly equal concentrations this assumption is only roughly true, as Hunter and Campbell [1917] and others have shown. The fault appears to lie chiefly, if not entirely, in the picric acid. All workers agree that the picric acid used in these determinations should be specially purified, and Folin and Doisy [1917] have given a somewhat elaborate method of purifying it. The most careful purification will not, however, make the above equation true, and the purest picric acid is liable to become impure if kept in solution. Hunter and Campbell have proposed to overcome the difficulty by reading the standard solutions against a large number of solutions of known creatinine content, and plotting the readings in a curve which is subsequently used in determining unknown

solutions. For accuracy this procedure would require repetition with each fresh lot of picric acid brought into use, and even with one lot if in use for more than a week or two, to make sure that it had not changed.

It was thought that a simple correction might be introduced into the above equation, on the assumption that the picric acid used contains a small amount of some substance which gives a red colour with alkali equivalent to so many milligrams of creatinine per 100 cc. If this amount is called p, and s and u are expressed in milligrams of creatinine per 100 cc., then the equation will run:

$$\frac{s+p}{u+p} = \frac{r}{10} \text{ and hence } u = \frac{10s}{r} + \frac{p\,(10-r)}{r}.$$

The value of p can be estimated for any sample of picric acid by reading two solutions of known creatinine concentration against each other, each being made up with the picric acid in question. Using the value of p thus found, standards made with this picric acid can be used for the determination of any unknown solutions which will give matchable colours. The purer the picric acid the smaller will be the value of p, but even with the purest picric acid p will not be so small that it can be neglected, except for a small range of unknowns which are nearly of the same concentration as the standard used.

In estimating the value of p for any lot of picric acid it is necessary to choose the concentrations of the two solutions to be compared suitably, or the error of the estimation will be very large. If the concentrations of the two solutions are called s and c, s being set at 10[1], then the value of p is given by

$$\frac{s+p}{c+p} = \frac{r}{10}; \text{ hence } p = \frac{10s - cr}{r - 10}.$$

The concentrations s and c should be chosen so that:

(1) they will give readings as far apart as is consistent with fairly matchable colours, and

(2) they shall be as small as is consistent with colours deep enough to read.

It was found that concentrations of 0 and between 0·2 and 0·3 mg. per 100 cc. met the above requirements.

To check the above reasoning a lot of saturated picric acid solution was made up from some "pure" picric acid, once re-crystallised, and its p estimated as follows.

A solution was made containing 0·2023 mg. of creatinine in 100 cc. of this saturated picric acid. 10 cc. of this and 10 cc. of the saturated picric acid itself were then taken and to each 0·5 cc. of 10 % sodium hydroxide solution was added, and the resulting colours read after standing ten minutes. The readings were as 10 : 19·5, which gives

$$\frac{0{\cdot}2023 + p}{0 + p} = \frac{19{\cdot}5}{10}; \text{ hence } p = 0{\cdot}21.$$

Standards containing 0, 0·25, 0·5 and 1·0 mg. of creatinine per 100 cc., made up with this lot of picric acid, had been read against each other and against other solutions of known creatinine content, and the readings observed

[1] It goes without saying that the standard may be set at any other more convenient figures 20, 30, etc. and these figures substituted for 10 in the equation.

and those calculated on the assumption that $p = 0{\cdot}21$ are shown in the following table.

TABLE.

Concentration of standard	Concentration of the other solution	Reading of the colorimeter	Calculated reading ($p = 0{\cdot}21$)
1·0	0·25	26·2	26·3
	0·33	23·0	22·4
	0·5	17·0	17·0
	0·67	13·8	13·7
	0·92	11·0	10·7
	1·0	10·0	10·0
	1·68	6·3	6·4
	2·33	5·1	4·7
	2·88	4·1	3·8
0·5	0·25	15·6	15·4
	0·5	10·0	10·0
	1·0	5·9	5·9
0·25	0·0	20·9	21·9
	0·25	10·0	10·0
	0·5	6·4	6·5
	1·0	3·8	3·8
0·0	0·0	10·0	10·0
	0·2	5·1	5·1
	0·25	4·8	4·6

It is suggested that a determination of this p affords a more accurate and useful guide to the purity of a sample of picric acid, than the test proposed by Folin and Doisy. In the latter test the colour of an alkaline picrate made by adding 5 % of 10 % sodium hydroxide solution to a saturated solution of the picric acid is compared with that of the picric acid itself. Their requirements of any sample to be used are that the colour of the picrate should not be more than twice as deep as that of the picric itself. This test is open to the objection that the colour of saturated solutions of picric acid, at the same temperature, varies considerably with different samples.

Two samples of picric acid were compared: (1) a solution which had been made a month previously of some acid re-crystallised from B.D.H. "pure," and (2) a freshly prepared solution of some picric acid purified by the method given by Folin and Doisy. Compared by Folin and Doisy's test the alkaline picrates of these gave readings of (1) 8·3, and (2) 8·6, against the respective acids at 20.

When the colours of the two solutions themselves, without the addition of sodium hydroxide, were compared, they read about 20 : 12·8 (number (2) being the lighter), but the colours were hardly matchable.

The value of p for the two samples, using in each case a 0 and a 0·2023 mg. per 100 cc. solution of creatinine in them, worked out at (1) 0·21, and (2) 0·10 respectively.

The thanks of the writer are due to Prof. R. A. Peters for the laboratory facilities afforded, and for the interest he has taken in the work.

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XLI. SOME OXIDATION PROCESSES OF NORMAL AND CANCER TISSUE.

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Since every difference between normal and cancer tissue may be of importance for cancer research, I compared some of the enzymic processes in normal muscle tissue with those of Jensen's rat sarcoma[1]. Furthermore the function of glutathione with the thermostable sarcoma residue was investigated on the same lines as those employed by Hopkins [1921, 1922] in the case of the thermostable muscle residue.

Methods.

The methods used for these investigations were those of oxygen uptake in a Barcroft apparatus of the form used in this laboratory and the methylene blue technique developed by Thunberg [1920].

A set of 4 Barcroft apparatus immersed side by side in the same water-bath was used. All were shaken by an electric motor at the same rate. The water-bath fitted with an efficient stirrer was kept in all experiments at $35° \pm 0{\cdot}01$. The flat-bottomed flasks of the Barcroft apparatus guaranteed a quick supply of oxygen. The reacting mixture was introduced into the right-hand flask, whilst the left bottle contained the same quantity of distilled water. Potash for the absorption of CO_2 was in every case placed in the receptacles provided. The oxygen uptake of the control experiment was determined simultaneously in a second apparatus. It is necessary, of course, to allow about seven minutes for the attainment of equilibrium in the bath before the flasks are connected with the manometer and the readings begun. Consequently, the oxidation had proceeded to a certain degree before the readings were commenced. In some experiments, however, the material to be oxidised was added after equilibrium was attained by using a special method. In this case an exactly quantitative measurement of oxygen uptake was possible. The calibration of the Barcroft apparatus was made by the simple and exact method related by Münzer and Neumann [1917].

All volumes are reduced to 0°, 760 mm. When not otherwise stated the reacting mixture had a p_H of 7·6 buffered with phosphate. In the methylene

[1] The strain used was one supplied to Prof. Hopkins by Dr J. H. Murray.

blue experiments after the solutions and the tissue had been added in the vacuum tubes all tubes were evacuated simultaneously by the same pump until the contained liquids boiled. Then the taps were closed and the tubes placed in a water-bath at 35°. The time of the beginning and of the end of the decolorisation was noted.

For a comparison with sarcoma a washed preparation from a normal tissue, especially rat muscle, was used; 6g. muscle of a freshly killed rat were cut with scissors during eight minutes, then generally washed three times for ten minutes each with a 50-fold bulk of distilled water, by using an electric shaker. After each washing the water was removed by pressing through linen. The sarcoma cannot conveniently be cut up in the same way. It was therefore pressed through wire gauze of 0·8 mm. mesh. The washing applied to the sarcoma was the same as for muscle.

For the experiments with glutathione the preparation was made in the manner described by Hopkins. The cut tissue was washed seven times with water. The first washing lasted only one minute, the second three minutes, and so on, increasing until one hour was used for the last washing. Then the tissue was boiled up, pressed through linen, treated with alcohol for 15 minutes and dried *in vacuo* over sulphuric acid, and finely powdered. This product in correspondence with the terminology used by Hopkins is here called the thermostable muscle- or sarcoma-residue.

Action of glutathione.

As Hopkins [1921, 1922] has pointed out, the thermostable muscle residue has only a small oxygen uptake when acting alone but a very large one if glutathione is added. The thermostable sarcoma residue gives the same results, no essential difference having been found. The figures of two comparable experiments with controls (1 *c*, 2 *c*) are quoted in Table I.

Table I.

Number of experiment ...	1	1 *c*	2	2 *c*
Muscle residue in g.	0·2 g.	0·2 g.	—	—
Sarcoma residue in g.	—	—	0·2 g.	0·2 g.
Phosphate $M/100$ p_H 7·6	0·2 cc.	0·2 cc.	0·2 cc.	0·2 cc.
Glutathione solution 0·8 % neutralised	1·0 cc.	—	0·1 cc.	—
Ringer's solution p_H 7·6	1·6	2·6	1·6	2·6
Cmm. O_2 taken up after 1 hour	119	37	152	29
,, ,, 2 hours	166	54	206	47
,, ,, 3 ,,	187	66	234	66
,, ,, 4 ,,	207	79	248	84
,, ,, 5 ,,	223	90	263	99

The phenomenon observed by Hopkins, that phosphate buffer in rather high concentration inhibits the speed and the total amount of oxygen taken up is also observed in the case of the sarcoma residue. A phosphate concentration of 1/15 molar in the reacting mixture for instance reduced the oxygen uptake to about the half.

Spontaneous oxidation of normal tissue.

Several authors have compared the oxygen uptake of normal tissue and cancer tissue. Russel and Gye [1920] as well as Watermann and Dirken [1921] found that tissue of a growth uses more oxygen and for a longer time than normal tissue. My experiments gave approximately the same results. It must be mentioned, however, that the results of different experiments are not quite consistent. Obviously the amount of disintegration suffered by the tissue has a notable influence on the speed of oxygen uptake.

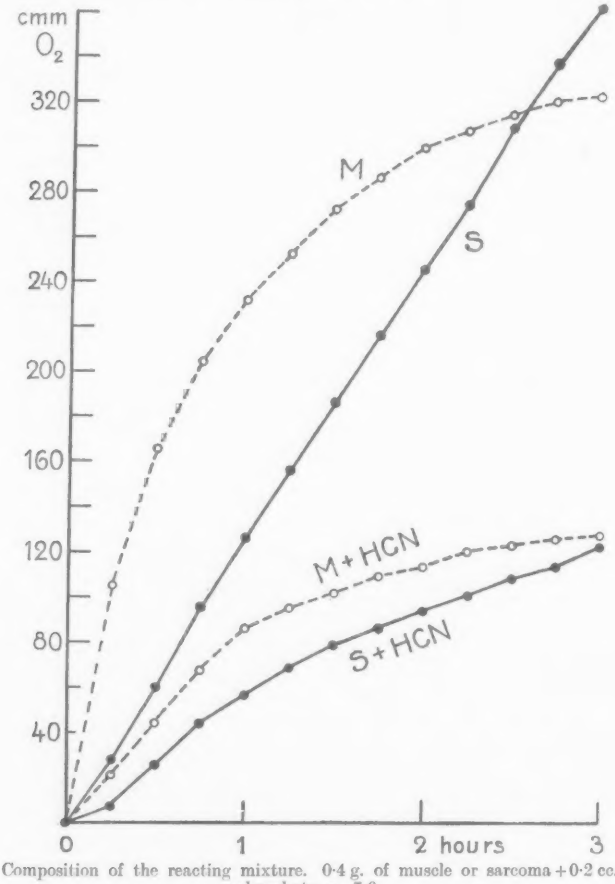

Fig. 1. Composition of the reacting mixture. 0·4 g. of muscle or sarcoma + 0·2 cc. of $M/2$ phosphate, p_H 7·6.

Curve marked	M	M + HCN	S	S + HCN
HCN $M/200$[1]	—	1·0	—	1·0
H_2O	2·4	1·4	2·4	1·4

[1] 0·5 cc. $M/100$ KCN was added and then neutralised by adding 0·5 cc. $M/100$ HCl.

In Fig. 1 the results of identical experiments are shown. The oxygen uptake of muscle tissue (curve *M*) shows great speed in the beginning, which soon becomes slower. The sarcoma (curve *S*), however, has an oxygen uptake which is slower at the beginning but at a constant rate during the whole course of the experiment, so that after three hours the sarcoma has absorbed more oxygen than the muscle. When the curve *S* is still rising the curve *M* has become nearly horizontal.

Furthermore the effect of cyanide was investigated and is illustrated in Fig. 1. It is evident that cyanide in a concentration of *M*/600 reduces the oxygen uptake in both cases, muscle as well as sarcoma, to approximately one-third.

The results from unwashed tissue obtained by the methylene blue technique *in vacuo* are shown in Table II.

Table II. 0·4 *g. of muscle or sarcoma* + 0·2 *cc. M*/2 *phosphate,* p_H 7·6 + 0·2 *cc. M*/2000 *methylene blue.*

	Muscle				Sarcoma			
Number of experiment	1	2	3	4	1	2	3	4
HCN *M*/200	—	0·6	—	—	—	0·6	—	—
HCN *M*/20	—	—	0·3	0·6	—	—	0·3	0·6
H_2O	0·6	—	0·3	—	0·6	—	0·3	—
Beginning of decolorisation in minutes	2·5	1·5	2·5	3	6	5	5	7
End of decolorisation in minutes	5	3	3·5	5	8	6·5	8	10

Comparing the methylene blue reduction by muscle (*M* 1) and sarcoma (*S* 1) we see that the muscle tissue reduced the methylene blue in 2·5–5 minutes, whilst an equal weight of sarcoma took 6–8 minutes. This result is in accordance with that of Drew [1920] and with those obtained with the oxygen uptake technique, where in the beginning muscle is more active than sarcoma.

But a surprising result is obtained in the experiments where cyanide is added. A relatively small concentration of HCN, *M*/467 in the experiments *M* 2 and *S* 2 produces an acceleration of the methylene blue reduction. Only in the presence of a very large cyanide concentration as in the experiments *M* 4 and *S* 4 is a small retardation of the reduction produced.

I should like to insist upon this result, that HCN produces an inhibition of the oxygen uptake but an accleration of the methylene blue decolorisation. Obviously the methylene blue technique of Thunberg is here not equivalent to the oxygen uptake method. Later on there will be an opportunity to discuss this difference further.

The oxidation of citric acid by washed tissue.

Citric acid added to a washed muscle tissue is oxidised. Battelli and Stern [1911, 2] have shown this by measurements of oxygen uptake and Thunberg [1920] by the methylene blue technique. Some results, consistent with many others, are shown in Table III.

As Thunberg pointed out, washed tissue alone may show a slow spontaneous reduction of the methylene blue. This "residual reduction" in Exp. *M* 3 (Table III) occupied 40 to 105 minutes in the case of muscle and 30 to 36 minutes in the case of sarcoma tissue (Exp. *S* 1). Citric acid added to muscle (*M* 2) produces a marked acceleration of the methylene blue reduction, so that the decolorisation begins after 13 and is finished after 30 minutes.

Table III. *0·2 g. muscle or 0·5 g. sarcoma + 0·2 cc. M/2 phosphate, p_H 7·6 + 0·2 cc. M/2000 methylene blue.*

	Muscle				Sarcoma			
Number of experiment	1	2	3	4	1	2	3	4
HCN *M*/100	—	—	0·2	0·2	—	—	0·4	0·4
Citric acid *M*/10	—	0·2	—	0·2	—	0·2	—	0·2
H_2O	0·6	0·4	0·4	0·2	0·6	0·4	0·2	—
Beginning of decolorisation in minutes	40	13	105	13	30	33	33	33
End of decolorisation	105	30	(360+)	48	36	55	70	100

The conditions of the experiments *M* 1 and *M* 2 being the same there can be no doubt that the citric acid is the material oxidised.

The experiments *M* 3 and *M* 4 show the influence of cyanide in the case of muscle. It is obvious that HCN (Exp. *M* 3) inhibits the residual reduction of the washed muscle tissue very strongly. This inhibition of the residual reduction was regularly observed in all experiments with washed muscle as well as with washed sarcoma. The difference between washed tissue and unwashed tissue as shown by the non-inhibition of the methylene blue reduction by cyanide in the case of the latter is noteworthy. Exp. *M* 4 shows that citric acid is also oxidised by muscle in presence of HCN. The reduction time is 13–48 minutes as compared with 105 to more than 360 minutes in the identical experiment *M* 3 without citric acid.

Working with sarcoma the results with citric acid are quite different (*S* 1–S 4 in Table III). It is very remarkable that citric acid in this case produces no acceleration of the reduction. Sometimes citric acid even inhibits the residual reduction of washed sarcoma. Thus in Exp. *S* 2 the time for complete decolorisation in presence of citric acid was 55 minutes as compared with 36 minutes without citric acid (*S* 1). Citric acid, as might be expected, has no effect when HCN is present (*S* 3 and *S* 4). This inefficiency of sarcoma in respect of the oxidation of citric acid was consistently found in many experiments. Sometimes the sarcoma was washed only once for some minutes instead of three times, sometimes the p_H of the reacting mixture was 6·0 instead of 7·6, the result being always negative. A different result was obtained with the oxygen uptake method as will be shown.

A further investigation with muscle dealt with the optimum p_H for the oxidation of citric acid. It was found with the methylene blue technique that the optimum is on the acid side at about p_H 6·0. For instance, the reduction times in a series of experiments with washed muscle and citric acid were 21–37 minutes at p_H 7·6; 12–21 minutes at p_H 6·5; 14–27 minutes at p_H 5·0.

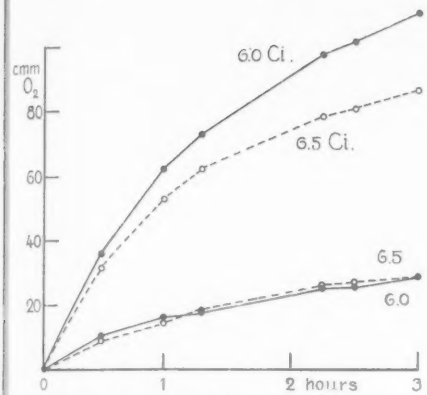

Fig. 2. Composition of reacting medium.

Washed muscle	0·5	·05	0·5	0·5
Phosphate *M*/2 p_H 6·0	0·2	0·2	—	—
Phosphate *M*/2 p_H 6·5	—	—	0·2	0·2
Citric acid *M*/10 neutral	—	0·2	—	0·2
H_2O	2·3	2·1	2·3	2·1

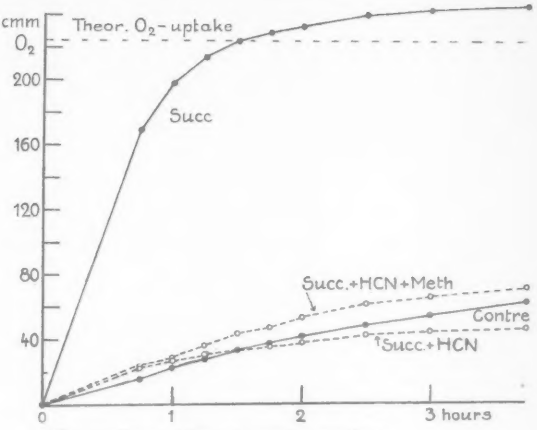

Fig. 4. Composition of reacting medium. 0·5 g. of sarcoma + 0·2 *M*/2 phosphate, p_H 7·6.

Succinic acid neutral. *M*/5	—	—	0·1	0·1	0·1
HCN *M*/100	—	—	0·3	0·3	
Methylene blue *M*/100	—	—	—	0·5	
H_2O	2·3	2·2	1·9	1·4	

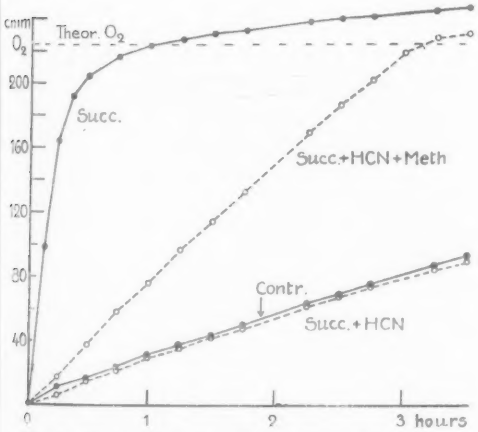

Fig. 3. Composition of reacting medium. 0·4 g. muscle + 0·2 cc. *M*/2phosphate, p_H 7·6.

Succinic acid neutral. *M*/5	—	0·1	0·1	0·1
HCN *M*/100	—	—	0·7	0·7
Methylene blue *M*/1000	—	—	—	0·5
H_2O	2·4	2·3	1·6	1·1

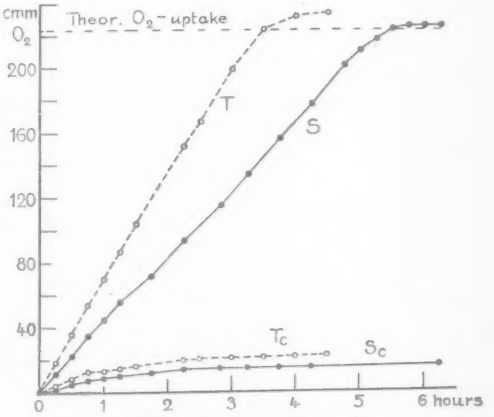

Fig. 5. Composition of reacting medium. 0·4 g. of muscle + 0·2 cc. *M*/2 phosphate, p_H 7·6 + 0·5 cc. *M*/100 HCN.

H_2O	0·8	0·9	1·3	1·4
Toluidine blue *M*/500	1·0	1·0	—	—
Soluble blue *M*/200	—	—	0·5	0·5
Succinic acid *M*/5	0·1	—	0·1	—

The same result was obtained with the oxygen uptake method. Working with washed muscle at p_H 7·6 the quantity of oxygen taken up by the citric acid is only very small. For instance, in an experiment at p_H 7·6 with citric acid 36 cmm. O_2 were taken up in two hours, while in the control experiment without citric acid 22 cmm. O_2 were absorbed in the same time. The rate of oxygen uptake at p_H 6·0 and 6·5 is much quicker as shown by Fig. 2.

Whilst both control experiments at p_H 6·0 and 6·5 show only the small O_2 uptake of 29 cmm. in three hours, the experiments with citric acid, and especially that at p_H 6·0 (full curve 6·0 *C* 1 in Fig. 2), show a good oxygen uptake.

Although sarcoma was found absolutely inefficient in oxidising citric acid by using methylene blue as a hydrogen acceptor, there is evidence that citric acid is oxidised by sarcoma when molecular oxygen is used. A thoroughly washed sarcoma does not show a distinctly higher oxygen uptake with citric acid than without it. But if the sarcoma is washed for a very short time only, say two minutes, then we get an unmistakable oxygen uptake due to the oxidation of citric acid. In one of the series, for instance, the control experiment took up 174 cmm. in $2\frac{3}{4}$ hours whilst the identical experiment but with citric acid took up 260 cmm. in the same time. Consequently 86 cmm. O_2 were due to the oxidation of the citric acid. But the addition of citric acid to preparations from the same individual sarcoma as that used in these experiments did not accelerate methylene blue reduction in the least.

The indications mentioned above, that the methylene blue technique of Thunberg is not equivalent to the oxygen uptake method, are thus extended.

Battelli and Stern long ago pointed out that the agent which oxidises citric acid is different from the enzyme oxidising succinic acid ("succinoxydase"). A tissue preparation loses its power to oxidise citric acid after standing for some hours, whilst the power to oxidise succinic acid remains constant for about two days. Moreover, the mechanism for the oxidation of citric acid is much less sensitive to HCN than is succinoxydase. I have observed this with the oxygen uptake method and also with the methylene blue technique. Washed rat muscle, for instance, to which citric acid had been added, reduced methylene blue when fresh in 13–30 minutes; after standing for four hours it required 40–115 minutes to reduce the same amount, while the data for the residual reduction (*i.e.* without citric acid) are 40–105 when fresh and 70–115 after four hours. After standing for 24 hours the decolorisation took place with and without citric acid in exactly the same time, *i.e.* 136–256 minutes. The oxidising power for succinic acid, however, was not diminished after 24 hours. Washing has a similar effect. The better a muscle preparation is washed the smaller becomes its power to oxidise citric acid, whilst washing may even raise the power to oxidise succinic acid. Furthermore, the oxidation of citric acid in the Barcroft apparatus is not inhibited by HCN *M*/1000, whereas the oxidation of succinic acid is completely suppressed by the same cyanide concentration. Moreover the optimum for the oxidation of citric acid is at p_H 6·0, whilst Ohlsson [1921] has found the optimum for the succinoxydase at p_H 8·7.

My results fully confirm those of Battelli, Stern and Thunberg in showing that the enzyme for the oxidation of citric acid is different from the succinoxydase. I have gone into this matter at some length because the difference between sarcoma and muscle in respect of the anaerobic oxidation of citric acid is a point of some interest.

Glutamic, lactic, malic, fumaric and maleic acids.

These acids were investigated mainly with the methylene blue technique using washed tissue. Glutamic acid was found to be the most effective oxygen acceptor with sarcoma as well as with muscle. The reduction time is always diminished (*M* 4 and *S* 4 in Table IV) when glutamic acid is added to tissue.

Lactic acid accelerates the methylene blue reduction if added to muscle. For instance, the reduction time with lactic acid (*M* 3 in Table IV) is 25–41 minutes whilst the residual reduction (*M* 1) takes 53–64 minutes. A somewhat different result is obtained with sarcoma.

Table IV. 0·2 *g. muscle or* 0·4 *g. sarcoma* + 0·2 *cc. M*/2 *phosphate*, p_H 7·6 + 0·2 *cc. M*/1000 *methylene blue.*

	Muscle				Sarcoma			
Number of experiment	1	2	3	4	1	2	3	4
Malic acid *M*/10	—	0·2	—	—	—	0·2	—	—
Lactic acid *M*/5	—	—	0·1	—	—	—	0·1	—
Glutamic acid *M*/5	—	—	—	0·1	—	—	—	0·1
H_2O	0·6	0·4	0·5	0·5	0·6	0·4	0·5	0·5
Beginning of decolorisation in minutes	53	25	25	16	23	40	20	15
End of decolorisation	64	*	41	41	29	*	29	23

* means that the decolorisation is not complete after 120 minutes.

Sometimes the acceleration of the reduction is distinct although very small, sometimes, however, lactic acid has no effect at all. There can be no doubt, that lactic acid is less effective with sarcoma than it is with muscle.

As regards malic acid, I observed when working with muscle that the decolorisation begins earlier (*M* 2, Table IV) but finishes later than the residual reduction. With sarcoma, however, malic acid was always found to inhibit the residual reduction.

Fumaric and maleic acids have never been found active in reducing methylene blue; on the contrary they regularly inhibit the residual reduction of washed tissue whether it be sarcoma or muscle.

The oxygen uptake method was also tried in the case of these acids, but the quantity of oxygen taken up was so small that clear results could not be obtained.

The oxidation of succinic acid and its mechanism.

The oxidation of succinic acid in the presence of washed muscle tissue has often been investigated, especially by Battelli and Stern [1911, 1] working with an oxygen uptake method, and by Thunberg [1918, 1923], Widmark [1921] and Ohlsson [1921] working with the methylene blue technique of

Thunberg. Battelli and Stern [1914] and later on also Widmark and Ohlsson worked with a muscle extract, in which the enzyme was in suspension. Einbeck [1919] confirmed the results of Battelli and Stern, by showing that one molecule of succinic acid takes up one atom of oxygen to yield fumaric acid. Fumaric acid in a second stage by taking up water is transformed into malic acid. In every case 25 % of fumaric acid remains unchanged.

Succinic acid was found to be a very powerful reducing substance for methylene blue if added to washed muscle. No other material investigated has a similar efficiency. In the experiment shown in Table V, succinic acid reduced 0·3 cc. of a *M*/1000 methylene blue solution in 4–18 minutes (*M* 2), whilst the reduction in the control without succinic acid (*M* 1) was not perceptible after 70 minutes. Cyanide (*M* 3) is seen to have no influence on this quick decolorisation.

Table V. *0·2 g. of muscle + 0·3 cc. M/1000 methylene blue or 0·3 g. of sarcoma + 0·2 cc. M/1000 methylene blue, with in each case 0·2 cc. of M/2 phosphate,* p_H 7·6.

	Muscle			Sarcoma			
Number of experiment	1	2	3	1	2	3	4
Succinic acid *M*/5	—	1	0·1	—	—	0·1	0·1
HCN *M*/100	—	—	0·2	—	0·3	—	0·3
H_2O	0·5	0·4	0·2	0·6	0·3	0·5	0·2
Beginning of decolorisation in minutes	*	4	4	33	33	16	16
End of decolorisation		18	18	83	200	37	83

* means more than 70 minutes.

HCN whether in small or in large concentrations (*M*/1000–*M*/50 have been tried) has no distinct effect upon the reduction due to succinic acid when added to washed muscle. An evaporation of the HCN by the evacuation of the tubes is certainly not responsible for this negative effect. In some experiments the tubes were not evacuated but the air replaced by hydrogen and the result was the same.

The effects obtained with sarcoma are somewhat different. First of all sarcoma is in the oxidation of succinic acid much less active than muscle. In the experiment *S* 3 of Table V the reduction time is 16–37 minutes as compared with 4–18 minutes for muscle (*M* 2), although more sarcoma and less methylene blue is used in the Exp. *S* 3 than in *M* 2. All results obtained with the methylene blue technique confirm this fact, that sarcoma has a much smaller oxidising power for succinic acid than muscle. A further difference exists in that cyanide appears to show an inhibitory effect on sarcoma. For instance, the reduction time in Exp. *S* 3 without HCN is 16–37 minutes against 16–83 minutes in *S* 4 with cyanide. This inhibitory effect of cyanide is however not quite constant; sometimes it was found to be more marked, sometimes less marked, than in the Exps. *S* 2 and *S* 4 of Table V. The difference may depend upon the degree to which the tissue has been deprived of its original reducing power by washing. If the inhibitory effect of cyanide be caused by a direct

action of HCN on the succinoxydase, *i.e.* the specific enzyme attacking the molecule of succinic acid, the fact would be remarkable since there is no similar effect of HCN upon the succinoxydase of muscle. It is possible on the other hand that the observed inhibitory effect of HCN may be due to the inhibition of the residual reduction of the washed tissue itself. As shown by Exps. *S* 1 and *S* 2 (Table V), and as I have indeed always found, the residual reduction is inhibited by cyanide. We must imagine that in Exp. *S* 3 of Table V only one part of the methylene blue is reduced by the hydrogen coming from succinic acid; the other part of the methylene blue is decolorised by the washed tissue itself. Consequently it is obvious that the reduction time must become longer if this residual reduction is diminished by cyanide. The residual reduction when sarcoma is concerned appreciably influences the observed reduction time since the succinoxydase of the sarcoma is not very active. This is not the case with muscle, where the active enzyme decolorises the methylene blue so quickly that the residual reducing power of the tissue can have only a slight influence. The complication due to the presence of two reducing factors (succinoxydase and residual reduction) prevents the forming of a definite conclusion. However, a quantitative comparison of all my experimental results seems to make doubtful any inhibition by cyanide on the succinoxydase of sarcoma.

Great activity of the succinoxydase is observed when succinic acid is shaken in the Barcroft apparatus with minced and washed rat muscle. In a typical experiment the control with muscle alone gave only the small oxygen uptake of about 80 cmm. in three hours (full curve Contr. in Fig. 3) while muscle and succinic acid together (full curve Succ. in Fig. 3) showed an oxygen uptake of 164 cmm. in the first 15 minutes. This extremely quick oxygen uptake was finished after one hour when about 220 cmm. O_2 had been absorbed. As Battelli and Stern and Einbeck have shown, succinic acid, $C_4H_6O_4$, when oxidised by tissues is transformed into fumaric acid, $C_4H_4O_4$.

One molecule of succinic acid therefore uses one atom of oxygen for the oxidation of hydrogen. The theoretical oxygen uptake (Theor. O_2 in Fig. 3) for the amount of succinic acid used in my experiments (0·1 cc. of a *M*/5 solution) should be 224 cmm. O_2 (reduced). This result has been obtained regularly in all experiments, showing that the method used is extremely reliable. Special precautions must of course be taken to avoid the oxidation of the succinic acid before the taps of the apparatus are closed. For this purpose the succinic acid was placed in small vessels in the flask of the Barcroft apparatus and only allowed to mix with the tissue in its buffer fluid after the taps were closed and thorough shaking had begun.

If cyanide be added to the system (tissue *plus* succinic acid) the rapid oxygen uptake disappears and we get a curve (dotted curve, Succ. + HCN in Fig. 3) which is nearly the same as that of the control (tissue alone). There can be no doubt that the oxidation of succinic acid is inhibited completely by cyanide. As to the effective concentration of cyanide it was found that

HCN in concentration of $M/3000$ in the reacting mixture inhibits the oxidation of succinic acid remarkably; $M/1500$ HCN prevents the oxidation completely.

The fact that cyanide inhibits oxygen uptake but not methylene blue reduction by succinic acid suggested experiments in which the influence of methylene blue upon oxygen uptake was studied. An addition of methylene blue to a flask containing muscle, succinic acid, and cyanide restores the oxidation of succinic acid as is shown by the dotted curve succ. + HCN + meth. of Fig. 3. It is certain that succinic acid is oxidised by this system, since the theoretical amount of oxygen (224 cmm.) is taken up. Furthermore this methylene blue effect is only seen in the complete system as used for the experiment "succ. + HCN + meth." in Fig. 3. The same combination but without muscle or without succinic acid takes up no oxygen. Methylene blue added to muscle plus succinic acid without cyanide never accelerates but rather inhibits the rate of oxygen uptake. The meaning of these experimental results will be discussed later on.

Similar experiments were carried out with sarcoma. A typical result corresponding to many others is shown in Fig. 4. Whilst the control experiment without succinic acid (full curve *contr.*) has only a small oxygen uptake, the addition of succinic acid (full curve *succ.* in Fig. 4) produces a quick uptake until the theoretical amount of 224 cmm. O_2 is absorbed. However, sarcoma never shows such velocity of oxygen uptake as muscle. Cyanide (dotted curve *succ.* + HCN) also inhibits the oxidation of succinic acid completely as it does with muscle. But there is this noteworthy difference that with sarcoma methylene blue scarcely restores the oxidation of succinic acid when inhibited by cyanide (compare Fig. 3 muscle with Fig. 4 sarcoma).

The quantitative relation between methylene blue reduction and oxygen uptake.

As I have pointed out already the methylene blue technique of Thunberg gives different results from the oxygen uptake method. This is especially the case when cyanide is applied. Apart from differences of a quantitative kind I have shown above that sarcoma oxidises citric acid in presence of molecular oxygen but not anaerobically in presence of methylene blue, while muscle of course oxidises citric acid in both cases freely. This might lead to the supposition, that the enzyme attacking the citric acid is different in sarcoma and muscle respectively. I would suggest however that a special factor exists responsible for the transport of hydrogen to methylene blue. We may speak of this as the "hydrogen transport factor." The assumption of such a hydrogen transport factor seems to me to be inevitable considering the quantitative relation between methylene blue reduction and oxygen uptake. This quantitative relation changes according to the treatment of the tissue. Some examples based upon investigations carried out with succinic acid will illustrate this fact.

Chopped rat muscle was washed twice, the first time for two and the second time for ten minutes. This muscle preparation "A" in oxidising succinic acid in the Barcroft apparatus gave rise to an initial speed of oxygen uptake corresponding to 212 cmm. in one hour. When the oxidation was wholly inhibited by the addition of sufficient cyanide and then so far as possible restored by addition of methylene blue, the speed of oxygen uptake due to the oxidation of succinic acid was 28 cmm. in one hour. The quotient of these two results, *i.e.*

$$\frac{\text{Cmm. } O_2 \text{ (succinic acid + HCN + methylene blue)}}{\text{Cmm. } O_2 \text{ (succinic acid)}},$$

which may be called the quotient meth. blue/O_2, gives a measure of the facility with which the hydrogen coming from succinic acid is transported to the methylene blue. A high value of the quotient meth. blue/O_2 means that this hydrogen is readily transported to the acceptor. It may be mentioned that the value of this quotient is nearly independent of the amount of succinoxydase present. This fact has been proved by using different amounts of the same muscle preparation.

In the experiment just mentioned the value is

$$\frac{\text{Methylene blue}}{O_2} = \frac{28}{212} = \frac{1}{7{\cdot}6}.$$

The muscle A was then washed once more for 2·5 hours. With this well-washed muscle, B, identical observations were made. The values obtained with muscle B were 264 cmm. O_2 for the direct oxidation of succinic acid and 14 cmm. O_2 when HCN and methylene blue were added to the system. Whilst the value for the direct oxidation rose from 212 to 264 cmm. the value for the oxidation by methylene blue fell from 28 to 14 cmm. Consequently the quotient meth. blue/O_2 fell from 1/7·6 to 1/19. This means that the facility of hydrogen transport to methylene blue is diminished by thorough washing but not transport to oxygen. The same results as described for muscle were obtained with sarcoma.

Identical results were also obtained by using Thunberg's technique where the direct oxidation of hydrogen is prevented not by cyanide but by the anaerobic conditions. In the experiment mentioned above in which the speed of oxygen uptake increased by washing from 212 to 264 cmm. the speed of methylene blue reduction in vacuum tubes decreased to about a quarter of its original value (reduction time for 0·3 cc. M/1000 methylene blue 36 minutes with the thoroughly washed muscle B against 10 minutes with muscle A).

Since I have made the same observation consistently in many experiments I conclude that a special hydrogen transport factor is responsible for the transport of hydrogen to methylene blue. The nature of such a factor in the reducing system is of course obscure. It would seem that continued washing with water removes it from the tissue or destroys it.

The quotient meth. blue/O_2 enables us to compare the activity of different organs in respect of the transport of hydrogen to methylene blue. I have

found for rat muscle washed in the usual manner values between 1/12 and 1/13; for frog muscle 1/12 (one experiment); for rat liver 1/20–1/24. Very unusual results were obtained in the case of sarcoma. The figures for this vary more widely than they do for muscle. Furthermore the figures obtained for sarcoma were with few exceptions very much smaller than for any other tissue. Values of 1/100 are quite usual. These strikingly small values for the quotient meth. blue/O_2 give an explanation for the phenomenon described above, that in the case of sarcoma methylene blue scarcely restores the oxidation of succinic acid when inhibited by cyanide. Apparently sarcoma possesses the hydrogen transport factor, which I have presumed to exist, in much smaller amount than other tissues.

Such a view may also explain the fact mentioned above, that citric acid with sarcoma does not reduce methylene blue though it takes up some oxygen in the Barcroft apparatus.

Whatever this hydrogen transport factor may be, its probable existence and its variability throw some light on Thunberg's methylene blue technique and its varying quantitative relation to the oxygen uptake method.

The oxidation mechanism of succinic acid as based upon activation of both hydrogen and oxygen.

The oxidation of succinic acid is a very proper object for investigations of the oxidation mechanism because we know important details of the chemical changes taking place. As Thunberg points out and Einbeck has proved, succinic acid losing two atoms of hydrogen is directly transformed to fumaric acid without addition of water. Thunberg therefore rightly calls this reaction the type of hydrogen activation and hydrogen transport in the sense of Wieland. It may be remembered that this hydrogen can be transported to methylene blue or to molecular oxygen as hydrogen acceptor.

Yet it seems to be extraordinary that this oxidation of succinic acid is inhibited by cyanide and restored by methylene blue. There can be no doubt that cyanide in small concentrations is a specific inhibitory substance for oxidation processes. Warburg [1921, 1923] considers that such oxidations depend upon catalysis by iron. The inhibitory effect of cyanide is due to the formation of a compound of HCN and iron; cyanide in fact inhibits the activation of oxygen by iron.

As to methylene blue, Meyerhof [1917, 1918] some years ago brought forward the hypothesis that this substance may be able to replace a normal factor in an oxidising mechanism when the latter has been inhibited or destroyed. Whilst the respiration of living micro-organisms is inhibited 10–40 % by methylene blue, the respiration of the same organisms treated with acetone and heated rises in the presence of methylene blue. The more the respiration is inhibited by heating, the stronger is the recuperative power of methylene blue. Furthermore Meyerhof showed that methylene blue can

restore to a certain degree the respiration inhibited by cyanide. In the same way Aschmarin [1922] considers methylene blue as an analogue to an oxidising enzyme.

Thunberg [1918] made the same observations as I, namely, that in the case of succinic acid cyanide does not inhibit the methylene blue reduction but only the oxygen uptake. Thunberg discusses the question as to whether cyanide might inhibit the enzyme succinoxydase, but the answer is a negative one, since the succinoxydase is able in presence of cyanide to transport the hydrogen to methylene blue. Therefore Thunberg inclines to the view that cyanide influences the oxygen, *i.e.* a hypothetical special form of oxygen, which alone can be directly used for the oxidation. As to the power of methylene blue to restore the oxidation inhibited by cyanide Thunberg gives no explanation, but he excludes a direct "inactivation" of the cyanide by methylene blue.

On the latter point my observations confirm Thunberg's view. I regularly found in my experiments that the amount of cyanide added is without influence upon the subsequent restorative power of methylene blue, so long as the HCN-concentration in every case be strong enough to suppress completely the direct oxidation of succinic acid. For instance two identical experiments containing the same amount of methylene blue, succinic acid and muscle show an oxygen uptake of 96 and 100 cmm. respectively, although the cyanide concentration in one experiment was $M/430$ and $M/100$ in the other. The restitution depends on the concentration of methylene blue only. I found that a five-fold amount of methylene blue approximately doubles the speed of oxygen uptake due to the oxidation of succinic acid in the presence of cyanide.

All the results discussed suggest the following theory concerning the oxidation mechanism of succinic acid. The specific enzyme which we may call, with Thunberg, "succino-dehydrogenase" (we reserve the name "succinoxydase" for another sense) induces a dehydrogenisation of succinic acid according to the formulae:

$$(1)\quad \begin{matrix} CH_2 \cdot COOH \\ | \\ CH_2 \cdot COOH \end{matrix} + \text{Enzyme} = \begin{matrix} CH \cdot COOH \\ \| \\ CH \cdot COOH \end{matrix} + (\text{Enzyme } H_2).$$

If the process takes place in the absence of oxygen but in the presence of methylene blue, the next step will be

$$(2)\quad (\text{Enzyme } H_2) + \text{methylene blue} = \text{Enzyme} + (\text{methylene blue } H_2).$$

The whole process is written here in two phases, although both reactions are probably coupled at the same surface. For the speed of the reaction (2) the "hydrogen transport factor" discussed in the previous section is of importance.

If the process on the other hand takes place in the presence of oxygen we shall have instead of (2) the reaction

$$(3)\quad 2\,(\text{Enzyme } H_2) + O_2 + X = 2\,\text{Enzyme} + 2H_2O + X.$$

In my opinion for this reaction (3) a catalyst X for the activation of

oxygen is necessary. This catalyst may be imagined to be an independent oxidising enzyme or it may be iron in the sense of Warburg.

All experimental results show that the reduction of methylene blue, involving the reactions (1) and (2), is not influenced by cyanide, which therefore inhibits neither the function of the enzyme "succino-dehydrogenase" nor the dissociation of the hydrogen from (enzyme H_2). On the other hand cyanide inhibits the reaction (3) completely. Consequently in the reaction (3) there must be a factor, *X*, necessary for this reaction which is sensitive to HCN. Since cyanide is known to be a specific inhibitory substance for oxidising enzymes or oxygen activators we may believe that *X* is an oxygen activator. We come then to the view, that the hydrogen of (enzyme H_2) cannot be oxidised by molecular unactivated oxygen. Consequently two enzymes are necessary for the oxidation of succinic acid, *i.e.* the specific enzyme "succino-dehydrogenase" which mobilises the hydrogen of the succinic acid molecule and the probably unspecific oxygen activator *X*. It will be pointed out later on that these two enzymes may in fact be only one enzyme with two different functions.

If in an aerobic experiment the oxygen catalyser *X*, and therefore the oxidation of succinic acid, is inhibited by cyanide, there exists a possibility of restoring this oxidation by adding methylene blue. Then the reactions (1) and (2) proceed in spite of the presence of cyanide, and the methylene blue is reduced. Since molecular oxygen is present the autoxidisable methylene white formed is continuously re-oxidised. Thus methylene blue replaces the catalyser *X*; it forms a substitute for an oxidising enzyme.

There are some other arguments to support this theory.

If the reaction (2) is in reality responsible for the power of methylene blue to restore the oxidation of succinic acid, then a parallel must exist between:

A. The power of a tissue to reduce methylene blue in presence of succinic acid *in vacuo*, and

B. The speed of oxygen uptake in an aerobic experiment with the same tissue in which the direct oxidation of succinic acid is inhibited by cyanide, but the indirect oxidation restored by addition of methylene blue.

It is doubtless somewhat difficult to compare the results of A with those of B, because in my experiments bearing on A no velocity curve was obtained but only the time necessary for the reduction of a certain quantity of methylene blue. Since however we only want relative figures the following procedure is sufficient. From the reduction time of the experiments done under the heading A the number of cmm. O_2 was calculated, corresponding to the amount of methylene blue which would be reduced in one hour (1 cc. $M/1000$ methylene blue is equal to 11·2 cmm. O_2). For the comparison we then take the quotient

$$\frac{\text{Cmm. } O_2 \text{ in 1 hour of experiment B}}{\text{Cmm. } O_2 \text{ in 1 hour of experiment A}} = \frac{B}{A}.$$

In 13 pairs of experiments with different tissues we got for B/A the following values:

Rat muscle, from 1·4 to 2·3; average 2·0.
Sarcoma, from 1·8 to 2·7; average 2·3.
Rat liver, from 2·1 to 2·3; average 2·2.
Foetal rabbit muscle (one experiment), 2·1.

Although the different experiments show a very marked difference in the actual power of methylene blue reduction, the values of the quotient B/A are very consistent. This is I think a strong argument in favour of the view, that, in the experiments of types A and B the same reaction (2) is taking place. Consequently the reaction (2), which is not inhibited by cyanide, is responsible for the power of methylene blue to re-establish the oxidation of succinic acid by a tissue under the influence of cyanide.

This theory involves the assumption that methylene blue is acting only as a hydrogen acceptor and that this function is responsible for the results observed. Lipschitz [1921, 1923] when describing his use of dinitrobenzene was inclined to minimise the significance of methylene blue reduction; but I quite agree with the view of Winterstein [1923] that every one of these reducible substances has its special advantages and disadvantages.

It seemed worth while however to decide whether other reducible substances have the same influence as that described for methylene blue. Toluidine blue, soluble blue and thionine were investigated to find out if they also can restore the oxidation of succinic acid when inhibited by cyanide. The experiments gave a clearly positive answer. Obviously there are differences in the activity, and methylene blue was found to be more active than the others. But all four substances are able to restore the oxidation of succinic acid by a tissue inhibited by cyanide. The theoretical oxygen uptake (224 cmm. for 0·1 cc. $M/5$ succinic acid) is ultimately reached in their presence. As soon as this is reached the oxygen uptake stops. In Fig. 5 two curves obtained with toluidine blue and soluble blue are shown.

In claiming that a "succino-dehydrogenase" and an oxygen activator X are necessary for the oxidation of succinic acid I have assumed that two distinct catalysts are actually present. It may be of course that the functions represented as separate in the scheme given above are in reality attached to one enzyme. If so it would be reasonable to name such an enzyme with the united functions "succinoxydase" in opposition to the hypothetical "succino-dehydrogenase" which was assumed to exert only one function. To settle this point an endeavour was made to find some treatment for the tissue which should destroy the factor X while leaving the succino-dehydrogenase intact. If this endeavour were successful we could prove the presence of the "succino-dehydrogenase" by restoring the oxidation with methylene blue. The different methods used were:

(1) Washed muscle was allowed to stand at room temperature for varying periods.

(2) Washed muscle preparations were heated to 50–56° for different lengths of time.

(3) Investigations were made at different hydrogen ion concentrations.

These and other attempts gave the same negative result. It was never possible to destroy or to diminish the activity of an oxygen activator without diminishing the "succino-dehydrogenase" effect at the same time. Consequently we think that only one catalyst, succinoxydase, is present, which unites the two different functions. Yet as has been shown the two functions are certainly different, since one only is inhibited by cyanide. If we look at the question from a biological point of view, it seems to be reasonable that only one enzyme should be present. Undoubtedly energy is used to split off hydrogen from the succinic acid molecule. On the other hand energy is produced by the oxidation of this hydrogen to water. It is probable that reactions so related are catalysed by one and the same agency.

At present a discussion is in progress as to whether the oxidation processes in the organism are caused by an "oxydase" system of the type described by Engler, Bach and Chodat, or by an activation and transport of hydrogen on the lines suggested by Wieland and Thunberg. The experiments described in this paper lead to the conclusion that the activation of both hydrogen and oxygen is necessary at least for the oxidation of succinic acid. Although it does not follow that this complex mechanism is necessary for all biological oxidations, it seems to me that this conception of a double activation (of hydrogen as well as of oxygen) may have significant bearings.

Summary.

A comparison was carried out between some oxidation processes due to normal tissue on the one hand and by sarcomatous tissue on the other. The methods used were measurements of oxygen uptake in the Barcroft differential apparatus and the methylene blue reduction technique of Thunberg in vacuum tubes.

(1) Glutathione has with the thermostable sarcoma residue the same effect as that found by Hopkins for the thermostable muscle residue.

(2) Unwashed sarcoma tissue reduces methylene blue more slowly than muscle. Likewise sarcoma exhibits a lower initial velocity of oxygen uptake than muscle; but the total amount of O_2 taken up weight for weight is greater for sarcoma tissue. Cyanide inhibits the oxygen uptake but not the methylene blue reduction of unwashed tissue, sarcoma as well as muscle.

(3) Citric acid accelerates the methylene blue reduction of washed muscle, but not of washed sarcoma. Cyanide has no influence. Muscle as well as sarcoma oxidises citric acid in the Barcroft apparatus. This oxidation is not sensitive to HCN; the optimum is at p_H 6·0.

(4) The methylene blue technique gave the following results with washed tissue. Glutamic acid is oxidised by muscle and by sarcoma. Lactic acid is less actively oxidised by sarcoma than by muscle. Fumaric, maleic and malic

acids have no distinct effect on the decolorisation of methylene blue by either tissue.

(5) Succinic acid is oxidised freely whether methylene blue or oxygen be the hydrogen acceptor. Muscle was found to be much more active in this respect than sarcoma. The quantity of oxygen used corresponds to one atom oxygen to one molecule succinic acid. Whilst HCN has no effect on the methylene blue reduction, the oxygen uptake by succinic acid is inhibited by cyanide. But the oxidation of succinic acid (with O_2) can in the case of muscle be restored if methylene blue is added to the system. This indirect oxidation of succinic acid induced by the methylene blue occurs to a much less extent, if at all, in the case of sarcoma.

(6) There is evidence that a "hydrogen transport factor" exists which is of importance for the transport of hydrogen to methylene blue. This "hydrogen factor" is distinct from the specific enzyme "succinoxydase." It can be washed out and its concentration is much smaller in sarcoma than in muscle tissue.

(7) The mechanism of the aerobic oxidation of succinic acid is investigated and discussed. The experiments lead to the assumption that the enzyme succinoxydase possesses two different functions: (*a*) the splitting off of 2H from the succinic acid molecule-activation and transport of 2H and (*b*) the activation of oxygen. The activation of oxygen as well as the activation of hydrogen is necessary for the oxidation of succinic acid.

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XLII. THE ANALYSIS OF PROTEINS. III. ESTIMATION OF HISTIDINE AND TYROSINE BY BROMINATION.

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Knoop [1908] described a colour reaction for histidine with bromine water in which bromine was apparently absorbed. Pauly and Gundermann [1908] obtained tri-iodoglyoxaline and Pauly [1910] *p*-nitrobenzoyl-di-iodohistidine, tetra-iodohistidine anhydride and di-iodohistidine anhydride. Pyman [1910] and Balaban and Pyman [1922] prepared the various bromo derivatives of glyoxaline. Though no bromo derivative of histidine has yet been made, these observations indicate that histidine would probably form a dibromo derivative. Histidine might also be expected to react in acid solution with bromate and bromide as was first shown by J. H. Millar [1903] in the case of tyrosine, and conversely by absorption of bromine it would be possible to estimate the histidine content of a solution. It has now been found that histidine absorbs two atoms of bromine by the above treatment, and, provided no other compounds which absorb bromine are present in the solution, it can be estimated by this reaction.

The application of this method to the estimation of histidine in proteins involves its separation from tyrosine and tryptophan, which also react with bromine. The separation from tyrosine is effected by precipitation with phosphotungstic acid. Its removal in this way was shown by Plimmer and Eaves [1913] to allow of the estimation of tyrosine. Tryptophan, though destroyed by acid hydrolysis of the protein, gives products which absorb bromine, and the tyrosine estimation was made after a short hydrolysis by trypsin. Fürth and Fleischmann [1922] have found that the decomposition products of tryptophan are removed by phosphotungstic acid and do not interfere with the tyrosine estimation. Their results were satisfactory, except in the case of keratin. The decomposition products of tryptophan are thus likely to interfere with the estimation of histidine. It was shown by Gortner and Blish [1915][1] that the nitrogen of tryptophan contributed largely to the humin nitrogen which is formed on acid hydrolysis. Our own experiments

[1] Further details on humin formation are given in Plimmer's *Chemical Constitution of the Proteins*, Part I. Third edition, 1917.

indicate that the decomposition products of tryptophan, which absorb bromine, are largely contained in the humin precipitated by lime, so that they may not interfere with the estimation of histidine. Unless arginine, lysine and cystine, which are also contained in the phosphotungstic acid precipitate, absorb bromine, the estimation of histidine thus becomes possible. Neither arginine nor lysine has been found to absorb bromine, but cystine has reacted, evidently by oxidation. The amount of cystine in proteins, except the sclero-proteins, is usually very small and cystine, moreover, is to a large extent decomposed by boiling with acids, as well as being incompletely precipitated by phosphotungstic acid [Plimmer, 1913]. The presence of cystine in the precipitated fraction is thus not likely to cause serious errors. Fürth and Fleischmann's high values for tyrosine in keratin were probably due to products of cystine decomposition in the phosphotungstic acid filtrate. Experiments have been made with gelatin to which known amounts of histidine, tyrosine and tryptophan have been added and they have been estimated with fair accuracy. Further work will be necessary to show that the method is applicable to other proteins and to ascertain the effect of the presence of cystine. The method has the advantage of giving a direct determination of histidine as a check on the indirect method by nitrous acid of Van Slyke [1911].

Experimental.

A. *Absorption of Bromine by Histidine and other Amino Acids.*

The bromination of histidine and other amino acids was carried out according to the procedure of Plimmer and Eaves [1913], in which an excess of *N*/5 sodium bromate and potassium bromide solutions is added to the solution of the amino acid in dilute hydrochloric acid. After an interval of 10 to 15 minutes excess of 4 % potassium iodide solution is introduced and the liberated iodine, corresponding to the excess of bromine, is titrated with standard sodium thiosulphate solution. In some experiments an excess of hydrochloric acid, or dilution with water, or a longer time of reaction, was tried, in order to test the absorption under different conditions. Exact details of the procedure are shown under the various experiments.

(1) *Histidine.* 0·506 g. of histidine hydrochloride, $C_6H_9O_2N_3 . HCl . H_2O$, was dissolved in 1000 cc. *N*/2 HCl. Portions of 100, 50 or 25 cc. were placed in a stoppered bottle. 10 cc. of 20 % potassium bromide were added, followed by *N*/5 sodium bromate solution. After 10 or 15 minutes 10 cc. of 4 % potassium iodide solution were quickly added and the liberated iodine titrated with standard sodium thiosulphate solution. The various quantities and the results are shown in the following table (p. 314).

Histidine thus reacts rapidly with bromine in acid solution, the reaction being complete in five minutes. The longer reaction time of 15 minutes is to be preferred. Excess of hydrochloric acid did not interfere.

Volume of histidine solution taken cc.	Volume of $N/5$ bromate solution added cc.	Titrated after mins.	Volume of thiosulphate solution required cc. $N/5$	Bromine absorbed g.	Theoretical amount of bromine for 2 atoms per molecule g.
100	20	15	17·5	0·0393	0·0386
100	20	120	17·3	0·0425	0·0386
100	10	5	7·45	0·0407	0·0386
100	10	10	7·45	0·0407	0·0386
			$N/10$		
50	5	10	7·65	0·0186	0·0193
50	5	10	7·30	0·0209	0·0193
50	5	15	7·20	0·0221	0·0193
25	5	10	8·70	0·0103	0·0097
25	5	15	8·65	0·0107	0·0097
50	5	15	7·50	0·0192	0·0193
50	5	15	7·40	0·0205	0·0193
			$N/20$		
25	5	15	17·10	0·0111	0·0097
25	5	15	17·15	0·0109	0·0097
50*	5	15	15·05	0·0195	0·0193

* 10 cc. of concentrated hydrochloric acid were also added.

The amount of bromine absorbed corresponds, as expected, with two atoms of bromine. The slightly higher values observed in most of the experiments are to be explained by a slight loss of bromine which occurs on removing the stopper from the bottle to introduce the potassium iodide solution. The results were also closer to the theoretical when $N/20$ thiosulphate was used; this strength is preferable on account of the small absorption of bromine.

(2) *Arginine.* 0·1556 g. of arginine carbonate was dissolved in 250 cc. of $N/2$ hydrochloric acid. 50 cc. portions were taken; 5 cc. $N/5$ sodium bromate, and 10 cc. 20 % potassium bromide were added. 10 cc. of 4 % potassium iodide solution were introduced and the solution titrated with standard thiosulphate of which 1 cc. = 0·2415 cc. bromate = 0·00386 g. bromine.

As seen in the following figures the amount of absorption of bromine by arginine is negligible, the small absorption of bromine being due to experimental error from escape of bromine in introducing the potassium iodide solution:

Vol. of arginine solution taken cc.	Titrated after mins.	Thiosulphate required cc.	Bromine absorbed g.
50	15	20·5	0·0008
50*	15	20·3	0·0015
50	15	20·45	0·0009
50	30	20·7	—

* 10 cc. of conc. HCl were also added.

(3) *Lysine.* 0·1563 g. of lysine picrate was dissolved in 250 cc. of $N/2$ hydrochloric acid. 5 cc. of $N/5$ sodium bromate, 10 cc. of 20 % potassium bromide were used for each determination, and 10 cc. of 4 % potassium iodide solution added before each titration.

1 cc. thiosulphate = 0·2415 cc. bromate = 0·00386 g. bromine.

Vol. of lysine solution taken cc.	Titrated after mins.	Thiosulphate required cc.	Bromine absorbed g.
50	15	20·65	0·0002
50	15	20·6	0·0004
50	15	20·5	0·0008

The amount of absorption of bromine is nil.

(4) *Cystine.* 0·3298 g. of cystine was dissolved in 250 cc. of $N/2$ hydrochloric acid. 50 cc. of the solution were treated with 20 cc. of $N/5$ sodium bromate solution and 20 cc. of 20 % potassium bromide solution. 10 cc. of 4 % potassium iodide were added before titration.

1 cc. thiosulphate = 0·9804 cc. bromate = 0·01567 g. bromine.

Vol. of cystine solution taken cc.	Titrated after hours	Thiosulphate required cc.	Bromine absorbed g.
50	0·5	6·9	0·2114
50	2·5	6·5	0·2178
50*	2·5	6·6	0·2162

* 10 cc. conc. HCl were also added.

A large amount of bromine is thus absorbed; it corresponds to 16 atoms of bromine per molecule of cystine, if allowance be made for slight loss of bromine during manipulation. The reaction which takes place appears to go to a final stage in half-an-hour. No experiments have been made to ascertain the products of the reaction, but from Friedmann's work [1902] the sulphur atoms are probably oxidised to sulphonic acid groups, with formation of cysteic acid.

(5) *Tryptophan and tyrosine.* Though it was found by Plimmer and Eaves [1913] and Homer [1915] that tryptophan absorbs bromine under the conditions described above, some further experiments were made to confirm these results.

0·57 g. of tryptophan was dissolved in 1000 cc. of $N/2$ hydrochloric acid. To 100 cc. of the solution 20 cc. of $N/5$ sodium bromate solution and 10 cc. of 20 % potassium bromide solution were added. At the end of the time of reaction 10 cc. of 4 % potassium iodide were added and the liberated iodine estimated with standard thiosulphate solution.

Vol. of tryptophan solution taken cc.	Titrated after mins.	Thiosulphate required $N/10$ cc.	Bromine absorbed g.
100	40	9·26	0·1715
100	90	8·95	0·1763
100	40	9·27	0·1712
50*	40	16·10	0·0615
50†	40	14·10	0·0936
100‡	40	11·10	0·1424
100‡	40	11·15	0·1376

* With the addition of 50 cc. of conc. HCl.

† With the addition of 150 cc. of water.

‡ After boiling with 50 cc. of conc. HCl under a reflux for 23 hours.

Again, there is a large absorption of bromine even after boiling with conc. hydrochloric acid for 23 hours. The amount of absorption represents from 6 to 8 atoms per molecule, the same amount as formerly observed.

Tyrosine was again found to be substituted by 2 atoms of bromine per molecule.

B. *Estimation of Histidine and Tyrosine in Proteins.*

Although cystine and the decomposition products of tryptophan absorb bromine, it seemed nevertheless possible to make estimations of histidine and tyrosine in proteins, if the products resulting from tryptophan were removed in the humin fraction and if proteins were used containing very little cystine, which is destroyed and incompletely precipitated by phosphotungstic acid.

In order to ascertain whether the method of bromination could be so applied, experiments were made with gelatin to which known amounts of histidine and tyrosine (*a*) singly, (*b*) in conjunction were added. Experiments were also made with the addition of tryptophan so as to find out if its products of decomposition influenced the results. Gelatin was used since it does not contain more than traces of tyrosine[1], tryptophan is not present in it, cystine[2] has not been isolated from it, and only 0·4 % of histidine has been obtained.

The general procedure of the hydrolysis and manipulations, was that of Van Slyke [1911]. The absorption of bromine was determined at each stage and at the same time the various kinds of nitrogen were estimated. 25 g. of gelatin, alone or with the addition of weighed amounts of the amino acids, were boiled under a reflux condenser with 85 cc. of 20 % HCl for 36 hours. The solution was evaporated *in vacuo* to a thick syrup, the syrup dissolved in water and diluted to 1000 cc. Total nitrogen was determined in a portion of 10 cc. and 100 cc. portions were taken for the bromination and for the nitrogen distribution estimations.

The total nitrogen estimations did not show the sum of the gelatin nitrogen and the added amino acid nitrogen, although the 25 g. were weighed out as exactly as possible. The N figure for the amino acid in each experiment was therefore calculated from the amount added and deducted from the total; the difference was then taken for the actual amount of the gelatin present. It was necessary to adopt this method, as the presence of these particular amino acids in the gelatin had to be allowed for in checking the estimation of the added quantities.

The amide and humin determinations were made in the usual way, but with the addition of a definite amount of lime, which in every case was 1·5 g. This modification has been found by Plimmer and Rosedale (unpublished results) to be preferable to the addition of an unknown excess of a suspension of lime in water; the amount necessary is calculated from the titration of the solution using phenolphthalein as indicator.

[1] Dakin [1920] gives 0·01 % as present in gelatin. [2] Sulphur is present [Dakin, 1920].

In those experiments for the determination of the bromine absorption (separate 100 cc. portions were here used) the filtrate and washings from the humin were evaporated *in vacuo* and made up to 200 cc. 50 cc. portions were then taken and treated with 10 cc. of $N/5$ sodium bromate solution (15 cc. in the second series) and 10 cc. of 20 % potassium bromide and 10 cc. of conc. HCl were added. After 15 minutes, 10 cc. of 4 % potassium iodide were quickly introduced and the liberated iodine titrated with $N/20$ sodium thiosulphate.

In the other experiments the filtrate and washings were acidified and precipitated with phosphotunsgtic acid according to the general procedure of Van Slyke.

The phosphotungstic precipitate was filtered off and washed with the same volume of wash liquid in each case. It was dissolved in very dilute NaOH and precipitated with $BaCl_2$. The filtrate from the barium phosphotungstate was made up to 200 cc. 50 cc. of the solution were used for each bromination, 10 cc. for total N, 40 cc. for arginine N and 10 cc. for amino N.

The solution containing the mono-amino acids was made just alkaline to phenolphthalein and treated with $BaCl_2$ to remove the phosphotungstic acid. The filtrate and washings (five times with 10 cc. of water) were evaporated *in vacuo* and made up to 200 cc. 50 cc. were taken for the bromination with 10 cc. $N/5$ bromate, 10 cc. potassium bromide and 10 cc. HCl and the excess bromine titrated after 15 minutes with $N/20$ sodium thiosulphate solution.

Table I. *Bromination Results.*

	Gelatin alone	Gelatin + histidine $HCl.H_2O$ 0·05 g.	Gelatin + tyrosine 0·1 g	Gelatin + tryptophan 0·045 g.	Gelatin + tyrosine 0·2 g.	Gelatin + tyrosine 0·2 g. + histidine $HCl.H_2O$ 0·1 g.	Gelatin + tyrosine 0·2 g. + histidine $HCl.H_2O$ 0·1 g. + tryptophan 0·099 g.
			After Amide and Humin				
Br absorption	0·0604	0·0988	0·2068	0·0836	0·3972 0·4144 0·4352	0·5360	0·6184
Br absorption of added amino acid	—	0·0384	0·1464	0·0232	0·3368 0·3540 0·3748	0·1204	0·0824
Corresponding wt. of amino acid	—	0·0504	0·0828	0·0077	0·1908 0·2004 0·2124	0·1480 (Histidine $HCl.H_2O$)	0·0270 (Tryptophan)
			Diamino Fraction				
Br absorption	0·0256	0·0536	0·0268	0·0408	0·0256	0·0920	0·1204
Br absorption of added histidine	—	0·0280	0·0012	0·0152	—	0·0664	0·0948
Corresponding wt. of histidine	—	0·0368	—	—	—	0·0868	0·1240
			Mono-amino Fraction				
Br absorption	0·0516	0·0628	0·2076	0·0616	0·2676	0·4140	0·3056
Br absorption of added tyrosine	—	0·0012	0·1460	—	0·2060	0·3524	0·2440
Corresponding wt. of tyrosine	—	—	0·0828	—	0·1172	0·1996	0·1384

By carrying out the bromination and the nitrogen distribution estimations in the same solution the results could be compared at each stage. They are given in Tables I and II, all data being calculated out to the original 100 cc. portion. The percentages of each variety of nitrogen are shown in Table III and the comparative values by the two methods are given in Table IV.

Table II. *Nitrogen Distribution Results.*

(Results are expressed in mg.)

	Gelatin alone	Gelatin + histidine $HCl.H_2O$ 0·05 g	Gelatin + tyrosine 0·1 g.	Gelatin + tryptophan 0·045 g.	Gelatin + tyrosine 0·2 g.	Gelatin + tyrosine 0·2 g. + histidine $HCl.H_2O$ 0·1 g.	Gelatin + tyrosine 0·2 g. + histidine $HCl.H_2O$ 0·1 g. + tryptophan 0·099 g.
Total N	370·4	380·7	377·9	370·7	383·5	399·8	415·7
N of added amino acid	0	10·1	7·7	6·2	15·4	20·2 15·4	20·2 15·4 13·6
Gelatin N by difference	370·0	370·6	370·2	364·5	368·1	364·2	366·5
Amide N	5·9	5·1	5·3	6·1	5·3	5·5	5·5
Humin N	2·9	3·3	3·5	6·6	1·6	1·6	5·5
Diamino							
Total N	119·1	124·7	109·2	117·6	113·8	118·1	115·8
Arginine N	58·0	40·0	61·6	56·0	61·0	64·0	59·0
Amino N	59·0	59·0	56·0	54·0	45·0	45·0	43·0
Non-amino N	60·1	65·7	53·2	63·6	68·8	73·1	72·8
Histidine N	24·8	53·6	10·5	32·4	34·6	37·7	42·8
Lysine N	36·2	31·1	7·1	29·2	18·2	16·4	14·0
Mono-amino							
Amino N	178·5	175·8	191·6	179·8	201·6	225·4	201·7
Non-amino N	57·6	59·3	53·3	54·4	66·8	54·8	75·7

Table III. *Nitrogen Distribution Results in Percentages of Total Nitrogen.*

	Gelatin alone	Gelatin + histidine $HCl.H_2O$ 0·05 g.	Gelatin + tyrosine 0·1 g.	Gelatin + tryptophan 0·045 g.	Gelatin + tyrosine 0·2 g.	Gelatin + tyrosine 0·2 g. + histidine $HCl.H_2O$ 0·1 g.	Gelatin + tyrosine 0·2 g. + histidine $HCl.H_2O$ 0·1 g. + tryptophan 0·099 g.
Total N	370·4	380·7	377·9	370·7	383·5	399·8	415·7
N of added amino acid	0	2·7	2·0	1·7	4·0	5·1* 3·9†	4·9* 3·7† 3·3‡
Gelatin N by difference	100·0	97·3	98·0	98·3	96·0	91·1	88·2
Amide N	1·6	1·4	1·4	1·6	1·4	1·4	1·3
Humin N	0·7	0·9	0·9	1·8	0·4	0·4	1·3
Diamino							
Total N	32·2	32·8	28·9	31·7	29·7	29·5	27·8
Arginine N	15·7	10·5	16·3	15·1	15·9	16·0	14·2
Amino N	15·9	15·5	14·8	14·6	11·7	11·3	10·8
Non-amino N	16·2	17·2	14·1	17·2	17·9	18·3	17·5
Histidine N	6·7	14·1	2·8	8·7	9·0	9·4	10·3
Lysine N	9·8	8·2	9·8	7·9	4·7	4·1	3·4
Mono-amino							
Amino N	48·2	46·2	50·7	48·5	52·6	56·4	48·5
Non-amino N	15·5	15·6	14·1	14·7	17·4	13·7	18·2

* Histidine. † Tyrosine. ‡ Tryptophan.

Table IV. *Comparison of the amounts of Histidine N and Tyrosine N found by the Bromination and Van Slyke methods.*

(Calculated in mg., making gelatin N 370·4 mg. in each estimation)

	Gelatin alone	Gelatin + histidine	Gelatin + tyrosine	Gelatin + tryptophan	Gelatin + tyrosine	Gelatin + tyrosine + histidine	Gelatin + tyrosine + histidine + tryptophan
Histidine N							
Bromination	6·7	14·4	7·1	10·7	6·7	24·2	31·7
Van Slyke	24·8	53·6	10·5	32·0	34·4	37·3	42·6
Histidine N added	—	10·1	—	—	—	20·2	20·2
Tyrosine N							
Bromination	2·7	2·7	10·1	2·7	11·7	18·1	13·4
Van Slyke (increase in amino N of mono fraction)	—	—	13·6	3·3	24·1	49·9	25·1
Tyrosine N added	—	—	7·7	—	15·4	15·4	15·4

On reference to the figures obtained by bromination (Table I) it is seen that the amount of histidine or tyrosine added was present in the solution after the amide and humin estimations. In the case of tryptophan, its products of decomposition which absorb bromine were almost entirely absorbed in the humin precipitate. The result shows that these products do not seriously interfere with the estimation of histidine or tyrosine, if they are removed by precipitation with lime. It may be possible on further trial to attain conditions in which this disturbing factor is completely eliminated. The correct weight of histidine was found in the first experiment, but in the second in which tyrosine was also present an excess of histidine was found. This excess was not found in the diamino fraction, and unfortunately cannot be accounted for. A small absorption, due to tryptophan products, was found in the experiment in which all three amino acids were present, and a slightly high result for histidine followed in the diamino fraction.

The results for histidine in the diamino fraction correspond very fairly with the amount added. Considering that the additional amounts were 2 and 4 % of the gelatin employed, the data can be looked upon as being within the region of experimental error, especially after the series of manipulations. Closer results should be expected with larger quantities of hydrolysed protein solution, or if more protein were taken in the hydrolysis.

The results for tyrosine were not so satisfactory. The result was low in the case of the addition of 0·1 g. and also in that of 0·2 g. One of these latter results was accurate. The tyrosine was not found in the diamino fraction. The two low values may be explained by insufficient washing of the barium phosphotungstate. Nitrogen was found in this precipitate on analysis by Kjeldahl's method. In the procedure adopted the excess of phosphotungstic acid was removed before bromination, but this is not necessary as was shown by Plimmer and Eaves and Fürth and Fleischmann.

The table of figures of the nitrogen distribution shows very erratic results. There was an increase in the humin nitrogen in those experiments in which tryptophan was added. The additional amount of nitrogen contained about 50 to 60 % of the nitrogen of the tryptophan. Additional histidine was found in all experiments except the one with tyrosine alone (0·1 g.). The data for tyrosine were also unsatisfactory. This inconsistency of the data by Van Slyke's method has been previously referred to by Plimmer [1916] and further work upon the cause is in progress. The bromination method as carried out with the Van Slyke procedure promises more accurate information about the histidine content of proteins.

It has not been possible to ascertain the effect of additional cystine; from Fürth and Fleischmann's results it would appear to interfere with the tyrosine estimation rather than with the histidine estimation.

If the bromination figure for the diamino fraction of gelatin alone is accurate, the amount of histidine in gelatin is 1·0 %. The tyrosine content works out at 0·85 %, a figure much higher than found by any other method. It is not considered to be correct.

It may be noted that in the estimation of 0·1 g. of tyrosine an amount of 0·0084 g. of nitrogen is involved, whereas 0·1765 g. of bromine is needed. The bromination method thus allows an error twenty times as great. In the case of histidine, 0·0271 g. of N is contained in 0·1 g.; 0·1030 g. of bromine is required for bromination; an error three times as great can then be allowed.

Summary.

1. Histidine reacts quantitatively with two atoms of bromine, and can be estimated in solution by bromination with bromate and bromide.

2. Arginine and lysine do not absorb bromine.

3. Cystine reacts with bromate and bromide, probably by oxidation.

4. The products of decomposition of tryptophan, which are formed on hydrolysis of proteins and absorb bromine, are almost completely contained in the humin precipitated by lime, and are thus separated from histidine and tyrosine.

5. Histidine can be estimated in the phosphotungstic acid precipitate of the hexone bases by bromination. Tyrosine can be estimated in the filtrate simultaneously. The presence of large amounts of cystine in the protein may, however, cause erroneous results.

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XLIII. THE ANALYSIS OF PROTEINS. IV. SOME ANALYSES OF GELATIN.

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In connection with the experiments of Plimmer and Phillips [1924] upon the estimation of histidine, after its addition to gelatin, by the method of bromination, some analyses of gelatin by Van Slyke's method [1911], carried out by us during the summer of 1919 at University College, are not without interest. The purpose of these analyses was to test the accuracy of the Van Slyke method with the addition of extra quantities of histidine to gelatin. At the same time the histidine content was estimated by the colorimetric method of Weiss and Ssobolew [1913]. In this way the two methods could be compared. By the latter method the presence of tyrosine is liable to lead to errors as it gives a similar colour with diazotised sulphanilic acid. Tyrosine was therefore also introduced to ascertain if it was completely removed from the phosphotungstate precipitate by washing. The colorimetric method was not used by Weiss and Ssobolew for estimating histidine in proteins, but has since been adapted for this purpose by Koessler and Hanke [1919, 1920] whose results did not appear till after our experiments had been completed. The desired accuracy has not been given by either method in our experience; further work in this connection to locate the source of error is in progress.

A valuable adjunct to the Van Slyke nitrogen distribution figures of proteins would be the determination of tyrosine in the mono-amino fraction. Its estimation is possible by bromination or by the colorimetric method of Folin and Denis [1912]. The bromination method was considered in Part III. The colorimetric method gives results which are higher than the gravimetric and are not considered to be accurate. The method has generally been applied to the hydrolysed protein directly and not to the fraction containing the mono-amino acids. If applied in this way very accurate results were obtained for amounts of tyrosine added to gelatin.

Experimental.

Seven analyses in duplicate of gelatin were made by Van Slyke's method; the first three were carried out on gelatin alone, the other four with the addition of histidine and tyrosine. In the first five experiments gold label gelatin was used, in the last two an English variety.

In each case about 8 g. of gelatin were hydrolysed by boiling with 20 parts of 20 % hydrochloric acid for 10 to 20 hours; after the hydrolysis, extra histidine was added in the form of the monohydrochloride, and tyrosine as such in those analyses in which they were introduced. The hydrolysed solution was evaporated *in vacuo* to a syrup, dissolved in water and made up to 250 cc. with water. Portions of 10 cc. were taken for determination of total nitrogen and two portions of 100 cc. each for the nitrogen distribution estimations. Except for the filtration of the hexone base phosphotungstate precipitate, the process was that given by Van Slyke. In Exps. 1 to 3 the filtration was carried out on an 8 cm. Buchner with a hardened filter paper, in Exps. 4 and 5 on one of 5 cm.; moderate suction was used. The quantity of wash liquid in Exps. 1 and 2 was not noted, but in the others it was 200 cc.; the time occupied in the filtration was from two to three hours; in Exps. 4 and 5 from four to five hours.

Arginine was estimated by the modification of Plimmer [1916].

The results of the experiments are given in Table I, corrections having been made for solubilities of the phosphotungstates, and the percentages of the different forms of nitrogen are given in Table II.

Table I. *Nitrogen Distribution Results.*

	I		II		III		IV				V			
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*c*)	(*d*)	(*a*)	(*b*)	(*c*)	(*d*)
	mg.		mg.		mg.		mg.		mg.		mg.		mg.	
Total N of gelatin	624·4		568·4		553·0		635·6		635·6		609·0		609·0	
N of added histidine	—		—		—		4·1		4·1	4·1	20·5		10·3	
N of added tyrosine	—		—		—		3·8		3·8	7·6	15·2		7·6	
Total N of experiment	624·4		568·4		553·0		643·5		643·5	647·3	644·7		626·9	
Amide N	10·6	10·5	10·1	9·2	8·3	8·8	5·9	10·1	9·9	9·4	17·9	17·6	16·8	16·1
Humin N	3·5	3·1	5·6	6·6	3·2	2·5	2·1	3·5	2·0	2·8	3·4	3·1	2·8	3·5
Diamino:														
Total N	145·6	134·4	134·4	121·8	127·4	124·6	170·8	159·6	190·4	179·4	159·8	173·6	147·0	156·8
Amino N	61·4	56·5	57·3	54·0	56·9	56·9	80·1	73·5	89·8	73·5	67·3	81·2	65·6	71·4
Arginine N	94·7	88·1	86·2	85·5	86·2	84·9	96·1	94·7	97·6	95·2	91·4	88·9	94·6	88·8
Histidine N	29·9	26·5	26·6	12·5	16·7	14·8	38·3	32·9	51·9	60·7	40·1	46·6	24·6	37·0
Lysine N	31·7	28·8	29·8	32·0	33·7	33·1	47·6	43·0	52·6	33·2	36·7	46·4	38·1	40·2
Mono-amino:														
Amino N	352·2	354·6	335·3	324·2	310·9	306·6	349·6	348·4	335·7	355·9	343·7	327·6	337·0	333·2
Non-amino N	91·6	131·4	104·6	115·2	99·5	102·3	118·5	125·4	123·8	124·4	99·1	109·4	108·4	101·9

As may be seen from the table of percentages, the data for amide nitrogen, except IV (*a*), were within the usual experimental errors; IV (*a*) was too low. The higher data for the experiments V were evidently connected with the variety of gelatin employed; in this case an English sample was used and it contained more amide nitrogen. The figures for humin nitrogen, except in experiment II, were fairly constant.

Table II. *Nitrogen Distribution Results in Percentages of Total Nitrogen.*

	I		II		III		IV				V			
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*c*)	(*d*)	(*a*)	(*b*)	(*c*)	(*d*)
Added histidine N	—		—		—		0·64		0·64	0·63	3·18	3·18	1·64	1·64
Added tyrosine N	—		—		—		0·59		0·59	1·17	2·36	2·36	1·21	1·21
Amide N	1·70	1·68	1·78	1·62	1·50	1·59	0·92	1·59	1·51	1·45	2·78	2·73	2·68	2·57
Humin N	0·56	0·50	0·99	1·16	0·58	0·45	0·33	0·54	0·27	0·43	0·53	0·48	0·45	0·56
Arginine N	15·17	14·11	15·17	15·05	15·59	15·36	14·94	14·72	15·17	14·71	14·18	13·79	15·09	14·17
Histidine N	4·79	4·24	4·79	2·20	3·02	2·62	5·95	5·11	8·00	9·38	6·24	7·23	3·92	5·90
Lysine N	5·08	4·61	5·24	5·63	6·09	5·99	7·40	6·82	8·18	5·13	5·69	7·20	6·08	6·41
Mono-amino N	56·39	56·82	58·99	57·05	56·22	55·46	54·34	54·16	52·17	54·98	53·31	50·81	53·75	53·15
Non-amino N	14·67	21·04	18·40	20·27	18·01	18·58	18·42	19·49	19·24	19·22	15·37	16·97	17·30	16·25
Total	98·36	103·00	105·36	102·98	101·01	100·05	102·30	102·43	104·54	105·30	98·10	99·21	99·27	99·01

The values for arginine nitrogen were on the whole very constant; with the gelatin alone one figure was lower than the remainder. The figures for histidine nitrogen were very irregular and did not show a proper increase in those experiments in which there was an addition. Considering that the lysine values are found by difference and that the histidine values are so erratic, it is remarkable that these data were so close to one another.

The mono-amino figures also showed great variations and no indication was shown of the addition of tyrosine by an extra amount of amino nitrogen.

On the whole, the data by the Van Slyke method cannot be looked upon as accurate, though they may give a good approximation. The irregularity may be connected with gelatin and may not be so great with other proteins, although great differences have also been found in their analyses. There is an indication that the error is in connection with the phosphotungstate precipitate; further work is confirming this preliminary observation.

The Colorimetric Estimation of Histidine.

Histidine was estimated in the above solutions of hydrolysed gelatin (except I (*a*), II (*a*), III (*a*) and (*b*)) according to the method of Weiss and Ssobolew [1913]. Special attention was given to those solutions to which histidine and tyrosine had been added, the object being to test the accuracy of the method. Tyrosine was added, as it gives a similar colour to histidine and it was of importance to ascertain if it was completely removed by washing from the precipitate of the phosphotungstates.

It was first necessary to determine the quantity of reagent which gave an optimal red colour with the standard histidine solution[1]. Using the reagents A and B of the same strength as described by Weiss and Ssobolew and a solution of histidine monohydrochloride ($C_6H_9O_2N_3$. HCl . H_2O) containing 1 mg. in 10 cc. (= 0·74 mg. histidine or 0·2005 mg. histidine nitrogen), it was found that the colour intensity was optimal with 0·3 cc. reagent (0·1A + 0· 2B) and 1 cc. of sodium carbonate in a total volume of 10 cc. and with 5 cc. of

[1] The method is described in Plimmer's *Chemical Constitution of the Proteins*, Part I, 3rd edition.

the histidine solution. These proportions were then used in the preparation of the standards for the colour comparisons.

Preliminary experiments were made with the various hydrolysed gelatin solutions in test tubes to ascertain the best dilution and quantity of reagent required to produce a colour of similar intensity to the above standard. A suitable quantity (1 or 2 cc.) was then diluted, mixed with the quantity of reagent and compared with the standard in a Duboscq colorimeter.

As gelatin contains a small amount of histidine, a colour comparison was made with the hydrolysed solution before the addition of the histidine hydrochloride in order to obtain the histidine content by this method. It was necessary for this purpose to assume that the tyrosine content of gelatin was negligible. The total histidine content of the solution was then determined in the diamino fraction as prepared by the Van Slyke method.

The data and calculations of the histidine content are shown in Table III. The values are calculated to a volume of 100 cc. hydrolysed gelatin according to the scheme

$$\frac{10}{11{\cdot}9} \times 10 \times 0{\cdot}2005.$$

In the case of the diamino solutions which were made up to a volume of 50 cc., from 100 cc. of original solution, the resultant figure is divided by two. A correction of 3·8 mg. has then been added for the solubility of histidine phosphotungstate.

Table III. *Colorimetric Estimations of Histidine.*

Experiment	Dilution	Colorimeter	Histidine N corr. for solubility mg.	Additional histidine N found mg.	Histidine N actually added mg.
I *b.* Diamino	1 : 10	10 : 11·9	12·1	...	...
II. Original	1 : 4	10 : 8·7	9·2	...	...
IV. Original	1 : 8	5 : 4·8	16·7	...	...
IV *a.* Diamino	1 : 10	5 : 4·5	14·9	− 1·8	4·1
IV *b.* „	1 : 10	5 : 4·4	15·2	− 1·5	4·1
IV *c.* „	1 : 20	5 : 5·2	23·1	+ 6·4	4·1
IV *d.* „	1 : 20	5 : 5·0	23·8	+ 7·1	4·1
V. Original	1 : 8	10 : 12	10·0	...	...
V *a.* Diamino	1 : 40	10 : 11	40·2	+30·2	20·5
V *b.* „	1·4 : 50	10 : 9	43·6	+33·6	20·5
V *c.* „	1 : 20	10 : 12·5	19·8	+ 9·8	10·3
V *d.* „	1 : 20	10 : 12·1	20·0	+10·0	10·3

The results were thus very variable. Only in the last experiment were data obtained which corresponded with the amount of histidine nitrogen added. The variations may be due to errors inherent in the complicated procedure of the colorimetric method, especially with the choice of the most suitable dilution to give the optimal red colour, or to some error with the preparation of the diamino fraction. Since this work was completed the adaptation of the colorimetric method to the estimation of histidine in proteins has been published by Hanke and Koessler [1920], who made special provision for the solubility of histidine phosphotungstate. In view of the erratic results

by the Van Slyke method, the main error would appear to be connected with the treatment of the phosphotungstate precipitate; the colorimetric procedure of Hanke and Koessler seems to be accurate.

The Colorimetric Estimation of Tyrosine.

Folin and Denis' method [1912] of estimating tyrosine has been tested by adding definite amounts of tyrosine to solutions of hydrolysed gelatin prepared in the same way as in the Van Slyke method. The several fractions were separated and tyrosine was estimated in each solution by the colorimetric method; from 1 to 3 cc. of the solutions were required to give a colour comparable with the standard. The figures in Table IV show that a loss occurs between the original solution and the solution obtained after removal of the humin nitrogen; it was not possible to recover this loss by prolonged washing of the humin. No loss was found in the phosphotungstate precipitate if this was sufficiently washed.

Table IV. *Colorimetric Estimations of Tyrosine.*

Experiment	Tyrosine found mg.	Tyrosine added mg.	Experiment	Tyrosine found mg.	Tyrosine added mg.
IV. Original solution	29·0	—	V *a*. Lime filtrate	187·0	200
IV *a*. Lime filtrate	57·0	50	V *b*. ,,	187·0	200
IV *b*. ,,	58·0	50	V *c*. ,,	105·3	100
IV *c*. ,,	57·0	50	V *d*. ,,	102·6	100
IV *d*. ,,	108·0	100	V *a*. Mono-fraction	182·8	200
IV *a*. Mono-fraction	59·5	50	V *b*. ,,	186·1	200
IV *b*. ,,	61·6	50	V *c*. ,,	114·8	100
IV *c*. ,,	52·7	50	V *d*. ,,	106·1	100
IV *d*. ,,	104·8	100	VI. Original solution	38·5	—
V. Original solution	28·6	—	VI *a*. Lime filtrate	75·2	50
			VI *b*. ,,	77·5	50

The loss that occurs between the original solution and the lime filtrate may in part be due to the removal of some substance giving the colour reaction and causing the high results generally obtained by the method. In all experiments except those with 200 mg. of tyrosine, the amount of tyrosine found has been slightly greater than the amount added. The estimation of tyrosine after removal of humin thus appears to give accurate results, if the solution does not contain more than 100 mg.

In order to avoid loss in the stage of removal of humin by lime, some further estimations were made omitting this step, but clearing the solution by means of phosphotungstic acid according to the Van Slyke procedure. In some experiments the solution and precipitate were made up to a definite volume and the estimation made in the mono-amino filtrate; in other experiments the phosphotungstic acid precipitate was filtered off and washed and filtrate and washings made up to a known volume. The results are here shown as percentages of tyrosine in gelatin, including the blank experiment figure in the amount of added tyrosine.

	Gelatin + tyrosine			Gelatin + tyrosine		
	Direct %	Filtrate %	Calculated %	Direct %	Filtrate %	Calculated %
I *a*	1·5	1·1	—	1·2	0·9	—
I *b*	1·5	1·1	—	—	0·9	—
II *a*	2·4	2·3	2·7	2·3	2·0	2·3
II *b*	2·4	2·3	2·7	—	2·0	2·3
III *a*	3·1	3·1	3·9	3·2	3·0	3·5
III *b*	3·4	3·4	3·9	—	3·0	3·5
IV *a*	5·1	4·7	5·0	—	4·0	4·7
IV *b*	5·2	4·7	5·0	—	4·0	4·7
V *a*	6·2	5·8	6·2	—	5·5	5·8
V *b*	6·2	5·8	6·2	—	5·2	5·8
VI *a*	7·5	7·4	7·4	—	6·2	6·7
VI *b*	7·6	—	—	—	6·2	6·7

By avoiding the lime precipitation the figures for tyrosine are thus very close to the calculated quantities; some interfering substance is removed by the phosphotungstic acid.

The same process was applied to the estimation of tyrosine in wool, caseinogen and egg albumin with the following results:

Caseinogen		Wool		Egg albumin	
	Phosphotungstic		Phosphotungstic		Phosphotungstic
Direct %	filtrate %	Direct %	filtrate %	Direct %	filtrate %
Using 1 cc. of solution:					
7·3	6·2	6·9	6·1	6·3	5·7
7·3	6·2	7·0	6·1	6·4	5·7
7·4	6·4	6·9	6·2	6·4	5·6
7·4	6·2	6·8	6·0	—	—
Using 2 cc. of solution:					
6·7	5·4	6·4	5·6	5·8	5·1
6·8	5·3	6·4	5·6	5·7	5·0
6·8	5·7	6·4	5·6	5·7	5·0
6·8	6·0	—	—	—	—
Using 2 cc. and filtering and washing phosphotungstates:					
6·5	6·2	6·4	6·0	6·0	5·5
6·5	6·2	6·4	6·0	6·0	5·3

The estimation of tyrosine can thus be carried out in the mono-amino fraction of the products of hydrolysis of proteins; the results are lower than those obtained directly. It is not possible to say whether they are more accurate; if there is an interfering substance it is removed by phosphotungstic acid.

Summary.

1. Duplicate analyses of gelatin by Van Slyke's method have given very erratic results.

2. Known quantities of histidine added to hydrolysed gelatin have not been accurately estimated.

3. The colorimetric method of estimating histidine has not given reliable results; the error seems to be in connection with the phosphotungstic precipitate.

4. Tyrosine can be estimated by the colorimetric method in solutions of hydrolysed proteins either after removing humin, or in the mono-amino fraction. The results are lower than by the direct estimation.

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XLIV. THE ISOELECTRIC POINTS OF SOME PLANT PROTEINS.

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Any attempt to define the living plant more precisely in terms of our present chemical or physical conceptions, is faced at the outset with the infinite possibilities which almost any given mass of living protoplasm apparently presents. The view, however, is generally accepted that many of the essential characters of living protoplasm are bound up with the peculiar properties of the proteins. While the protein properties in general are possibly better known than are those of the individual plant proteins, a number of isolated facts suggested to us that the typical plant protoplasm, although probably different chemically in different genera of plants, tended to show in nature a common reaction to the external medium and behaved as though possessing a negative electrical charge. The facts, moreover, appeared to be most readily explained on the assumption that the protoplasm was composed largely of proteins, and it followed as a natural result of Loeb's work [1922] that these proteins were in nature commonly on the alkaline side of their isoelectric points. The typical plant parenchymatous tissues have usually a sap reaction of about p_H 5·5–6·5. The isoelectric points of plant proteins appear to be almost entirely unknown. The following investigations were therefore undertaken to ascertain isoelectric points for a series of representative proteins.

Methods.

It is apparent from this brief introduction that our investigation must take the nature of a preliminary survey over a fairly wide field. We should have to include representatives of the typical groups of vegetable proteins, the globulins, albumins and prolamins, and since the majority of known vegetable proteins are reserve substances, it was obviously necessary to include also some which were undoubtedly physiologically active (*e.g.* leucosin and tuberin). The relatively simple plants, *Nitella* and yeast, were included because direct determinations on protoplasm (as distinct from extracted proteins) seemed possible in these cases. It is obvious that this widening of the field of enquiry must profoundly influence the methods adopted. The isoelectric point of an ampholyte is usually determined by cataphoresis [see

Burton, 1916] or by the precipitation method [well discussed by Cohn, 1922]. Cataphoresis is, however, not always possible, particularly in the case of the prolamins or of such proteins as glutenin and caseinogen, which are not soluble in water or salt solutions [Cohn, 1922] and this, in itself, suggested that the precipitation method would prove more generally useful in survey work such as ours. In addition, since plant proteins are not as a rule well known or well defined, it was anticipated that relatively crude products would often represent the only material available, and it was desired to consider the behaviour of crude extracts in order to consider the possibility of determinations of isoelectric points in cases where the proteins were unknown, or occurred in mixtures. (The bean globulins are taken in illustration of this point.) Under such circumstances the precipitation method and colorimetric determinations of hydrogen ion concentration appeared to be both more generally useful and more rapid, and refinement of technique seemed to be of subordinate importance. Our purpose is clearly better served by finding the approximate position of the isoelectric points of a number of proteins than by very accurate determinations of the isoelectric points of one or two. We do not wish to imply that either our results or precipitation methods are inaccurate. A number of other methods have been employed in these investigations which give concordant results, although reasons of space prevent their discussion here. Further, we examined tuberin and edestin and obtained the same results by our methods as other workers had previously obtained using cataphoresis and electrometric determinations of hydrogen ion concentration.

The details of the methods used are as follows. Ten or twenty cubic centimetres of a given protein "solution" were taken in each of a series of graduated tubes of uniform bore. The solutions were acidified or made alkaline to varying degrees and made up to uniform volume with distilled water, since in the case of salt extracts, the degree of precipitation would be affected by the degree of dilution of the original salt solution. They were then allowed to stand and at a fixed time after treatment, usually half-an-hour, were centrifuged on an electrically driven centrifuge (at 1500 revolutions per minute) for equal periods, usually of two minutes. The volumes of the precipitate were then measured, or if the precipitates were of markedly variable consistency, they were dried and weighed. Centrifuging served to eliminate the marked differences in the rate of sedimentation. It is probably essential in many cases, as on allowing the precipitates to settle slowly some may show marked changes in volume in periods of a few hours, probably owing to hydrolysis of the protein by acid or alkali. An example of this is discussed under the heading of bean globulins.

To bring the protein solutions to the required reactions, the following reagents were used:

(1) Hydrochloric acid or sodium hydroxide.
(2) Acetic acid or sodium hydroxide.
(3) Citric acid or disodium phosphate.

The last pair form buffer solutions of a sufficiently wide range for most biological purposes [McIlvaine, 1921]. It is important to use the same proportions of salt in all determinations and to vary the acid only, as the solubility of a protein is intimately connected with the salt content of the solution. Cohn [1922] has shown, however, that, if the salt content is constant, the apparent position of the isoelectric point is not significantly affected.

Although the results given are only those for one of the reagents mentioned above, the three sets have been used in most cases, the results being similar to those given. Only the methods stated were employed for leucosin, gliadin and *Nitella*.

In the majority of cases, where a single protein can be extracted in aqueous or salt solution, the precipitation method offers no great difficulty. We have employed both crude extracts and "purified" proteins—with the same results. In the crude extracts the maximum precipitation is usually less sharply marked however. When the tissue extract contains two or more proteins or their derivatives, difficulties may arise. Such a case is that of the bean globulins discussed below. A further difficulty is presented by leucosin (from wheat) which is not readily precipitated by acid or alkali. A modified method has been used in this instance.

In view of the large number of estimations required in a given series of observations, the colorimetric method of determining hydrogen ion concentration was employed with the usual precautions [Clark, 1920]. This involves the possibility of a "protein" error. Since the solutions employed were normally quite clear, and since a limited number of check readings with the hydrogen electrode gave concordant results, the error at or near the isoelectric point of the protein is probably not important. The "salt" error in colorimetric determinations does not affect the results since most of our estimations were made in the presence of minimal and similar quantities of salts. It may be noted that the results in the presence of the phosphate buffer solutions were not significantly different from those obtained by other methods.

Results.

1. *Broad Bean* (Vicia faba).

As in most leguminous seeds, the two chief proteins (globulins) in the cotyledons of broad bean are vicilin and legumin. These storage proteins are readily extracted from ground seeds in 10 % sodium chloride solution. The extract is filtered off and saturated with ammonium sulphate crystals, the globulins being then precipitated. They are washed and redissolved in dilute ammonium sulphate (1/100 saturation). Saturated ammonium sulphate solution is then added until the whole solution becomes 6/10 saturated in respect of this salt, when legumin is precipitated. It is purified by repeating the process and finally separating by dialysis. The filtrate from legumin is saturated with ammonium sulphate—when vicilin separates. This is purified

by repeating the whole process of solution, 6/10 and complete saturation and is then separated by dialysis. Vicilin in 1 % sodium chloride or dilute ammonium sulphate shows maximum precipitation, on acidifying, at p_H 3·4 or a little higher. Legumin in similar solutions shows maximum precipitation at p_H 4·6. These results are illustrated in the following table:

Table I.

Vicilin in 6/10 saturated ammonium sulphate solution			Legumin in 1 % sodium chloride solution		
Acid added cc. of *N* HCl	Precipitate cc.	p_H	Acid added cc. of *N* HCl	Precipitate cc.	p_H
0·1	1·4	5·0	0	0	5·2
0·2	1·7	4·4	0·1	0·5	4·8
0·3	2·3	3·8	0·2	1·0	4·7
0·4	2·7	3·4	0·3	1·3	4·6
0·6	1·7	3·2	0·4	1·15	4·4
0·8	1·6	3·0	0·6	1·0	4·0
1·0	1·3	2·9	0·8	0·9	3·6
1·2	1·1	2·8	1·0	0·8	3·2
0	0	5·3	1·2	0·6	2·8

The same results were obtained using suspensions of the proteins in water. In this and subsequent tables where no values are given for the higher p_H values, little or no precipitation occurs up to p_H 9·0.

Crude extracts of bean globulins. In view of the possibility of having to examine unidentified proteins in relatively crude extracts, the behaviour of the bean globulins in crude extracts with 10 % sodium chloride solution was considered. The fresh extracts of ground seeds were filtered to remove starch and other suspended matter, and then made acid or alkaline in the usual way. If the solutions were centrifuged immediately (within five minutes) after changing the reaction, maximum precipitation occurred at p_H values of 4·6 and 3·4. The precipitation curve apparently shows the effects of both globulins, vicilin and legumin, when this method of procedure is adopted (Table II, I).

If the solutions are allowed to stand for 30 minutes or more after changing the reaction, maximum precipitation takes place at much higher hydrogen ion concentrations, particularly about p_H values of 3·3 and 2·0, and the point in the precipitation curve at p_H 4·6 almost disappears (Table II, II).

A somewhat similar result is obtained on allowing the precipitate obtained by centrifuging immediately after acidifying to stand for some time (6 to 24 hours). It is then found that the volume of the precipitate at p_H 4·6 tends to decrease, while much further precipitation occurs in the solutions of higher hydrogen ion concentration. It seems possible that hydrolysis of the legumin takes place on standing and that the decomposition products have isoelectric points at lower p_H values. Vicilin, however, appears to be less affected by this treatment and may possibly be derived from legumin. In dealing with crude extracts it seems advisable to centrifuge and measure the precipitate

immediately on changing the reaction, to avoid the possibility of hydrolysis of less stable proteins.

The proteins of the crude extracts may be readily separated by employing the fact that their isoelectric points occur in solutions of different p_H value. The crude extract is poured into excess of water acidified to give the required final p_H value. The precipitate can be filtered off, redissolved or suspended and its point of maximum precipitation determined. The figures obtained for legumin in this way show a rather lower p_H value (4·4) for the isoelectric point, while vicilin gives the same result, as when using the purified proteins (Table II, III, IV).

Table II. *Extracts of globulins from bean cotyledons.*

I		II		III		IV	
p_H	Precipitate in cc.	p_H	Precipitate in cc.	p_H	Precipitate in cc.	p_H	Precipitate in cc.
5·8	0	7·4	tr.	8·0	0	6·5	0
5·0	3·0	6·2	tr.	7·0	tr.	5·2	0·4
4·6	**4·2**	5·4	tr.	5·0	0·5	4·7	1·2
4·4	2·3	4·9	0·8	4·2	0·9	4·6	1·5
4·2	3·2	4·6	2·2	3·8	1·3	**4·4**	**3·7**
4·0	3·7	4·5	2·0	3·6	2·8	3·6	0·7
3·8	4·0	4·4	2·6	**3·4**	**5·3**	3·4	0·7
3·6	4·3	4·2	4·0	3·2	1·2	—	—
3·4	**4·8**	**3·3**	**5·2**	3·0	0·8	—	—
2·2	3·1	2·6	3·0	2·8	0·4	—	—
1·6	2·8	2·4	5·5	—	—	—	—
—	—	**2·0**	**6·0**	—	—	—	—

20 cc. of filtered sodium chloride solution taken; hydrochloric acid and sodium hydroxide used to change reaction.

I. Centrifuged immediately after treatment.

II. Centrifuged 30 minutes after treatment.

III. Redissolved precipitate after precipitating in excess acidified water at p_H 3·6 (vicilin).

IV. Redissolved precipitate after precipitating at p_H 4·7 (legumin).

Proteins of growing beans. The possibility exists that the physiologically active proteins of living tissues bear little or no relation to the reserve proteins. It seemed of interest, therefore, to attempt to determine the isoelectric points of the proteins found in growing beans. Using both aqueous and salt extracts we have only been able to obtain very small quantities of precipitable protein in extracts of fresh tissues. While it appears to be safe to say that on acidifying these extracts most precipitation occurs *near* p_H values of 3·4 and 4·5, the volumes of the precipitates are so small that nothing more precise can be stated. We hope to consider further the question of dealing with the physiologically active proteins in subsequent papers.

2. *Pea* (Pisum sativum).

The globulins, vicilin and legumin, obtained from peas behave in the same way as those from broad beans. Both seeds contain rather small quantities of an albumin, legumelin. We obtained this most readily from pea flour, by dialysing an aqueous extract until the globulins were precipitated, adding

from time to time a few drops of sodium hydroxide to keep the solution neutral. The solution is then filtered and the filtrate acidified, and the samples heated simultaneously to 45–50°.

Table III. *Precipitation of legumelin from* 20 *cc. of aqueous solution.*

cc. of *N* HCl added	p_H	Precipitate in mg.	cc. of *N* HCl added	p_H	Precipitate in mg.
0	6·8	0	1·2	**4·1**	**167**
0·1	6·4	0	1·5	3·9	57
0·3	5·9	107	2·0	3·6	0
0·5	5·1	119	3·0	3·1	0
0·7	4·7	150	4·0	2·7	0
1·0	**4·3**	**163**			

Legumelin obtained from broad beans behaved in a similar way. Maximum precipitation occurred about p_H 4·1–4·3 and the isoelectric point appears to be close to p_H 4·2. The difficulty about legumelin is, however, the uncertainty as to whether the first product is free from the globulin legumin or not, since the latter is also slightly soluble in water. Dialysis cannot be continued very long owing to the ease with which hydrolysis takes place.

3. *Wheat* (Triticum vulgare).

This was taken as being typical of the cereals. In wheat the two principal reserve proteins are glutenin and gliadin, both being present to the extent of about 4 %.

Glutenin was prepared by extracting ground wheat seeds several times with warm alcohol (90 %) to remove the gliadin, and then extracting with 0·1 % potassium hydroxide. The glutenin may be precipitated from this solution with acetic acid and purified by washing, redissolving and precipitating again. This standard method of preparation obviously depends on precipitation at the isoelectric point. We varied it by dissolving in 5 % acetic acid and precipitating with sodium hydroxide. Both solutions in acetic acid and in sodium hydroxide gave maximum precipitation at p_H 4·4–4·5. In the sodium hydroxide solution a broad zone of high precipitation occurs between p_H 4·1 and 5·0. This may be characteristic of glutenin or may be due to the presence of another amphoteric substance, for on dissolving glutenin in 0·2 % acetic acid (to obtain a solution about p_H 4·5) and then making alkaline, a slight precipitate about p_H 5·5 was obtained. It is doubtful whether this is protein. A minor precipitation maximum also occurs about p_H 3·6. The precipitate at this point is largely protein.

Typical results are embodied in Table IV.

Gliadin is present in the extract of wheat with 90 % alcohol. The solution after concentration by boiling, preferably under reduced pressure, is poured into water, when the gliadin is precipitated. It is purified by dissolving in 90 % alcohol and precipitating again.

Table IV. *Glutenin.* 20 *cc. of solutions taken.*

(*a*) 0·2 % NaOH solution			(*b*) 5 % acetic acid solution			(*c*) 0·2 % acetic acid solution		
Acid added cc. 5 % acetic	Precipitate cc.	p_H	Alkali added cc. 4 *N* NaOH	Precipitate cc.	p_H	Alkali added cc. *N*/10 NaOH	Precipitate cc.	p_H
0	0·2	10·2	0	0	3·2	0	0	4·6
0·6	1·0	8·0	0·2	**2·7**	**3·6**	2·2	0	4·7
1·0	2·8	6·9	0·4	1·2	3·8	2·6	0	4·8
1·4	3·3	5·0	0·6	1·6	4·0	3·0	0	—
2·0	3·5	4·8	0·8	2·6	4·2	3·4	tr.	4·9
3·2	3·7	4·6	1·0	2·0	4·3	4·0	0·2	5·0
3·6	3·8	4·5	1·2	**3·4**	**4·4**	4·6	**0·4**	**5·5**
4·0	**4·0**	**4·4**	1·4	**3·4**	**4·5**	5·4	0·2	5·8
4·4	3·7	4·3	1·6	1·8	4·6	6·0	tr.	6·2
5·0	3·7	4·2	1·8	1·6	4·8	6·8	0	9·6
6·0	3·0	4·0	2·0	1·2	5·0	—	—	—
8·0	2·4	3·4	2·2	—	6·2	—	—	—

McClendon [1917] gives the isoelectric point of gliadin as p_H 9·3 on the authority of Michaelis. We have no evidence in support of this. Gliadin is soluble in dilute alkalies or acids. On neutralising these solutions, gliadin is precipitated—but the precipitates appear to be of approximately equal volume over a rather wide range of p_H values (3·0–7·0). On pouring an alcoholic solution of gliadin into ten times the volume of water previously made acid or alkaline with HCl or KOH, a fine precipitate of the gliadin appears rapidly in solutions more acid than p_H 5·5, and the maximum turbidity lies between p_H 3·5 and p_H 5·5. The isoelectric point probably lies between these limits.

A series of equal volumes of solution of gliadin in 80 % alcohol was acidified or made alkaline with *N*/50 HCl or NaOH, each of the samples being made up to the same volume with distilled water, so that the volume of the alcoholic solution was four times that of the added acid and water. (The alcoholic solution thus became a 64 % alcohol solution.) Using this procedure, maximum precipitation was obtained about p_H 5·2, though the volume of protein precipitated was small. Though larger precipitates can be obtained by diluting further there is of course the danger of precipitating all the protein, which is insoluble in water.

Table V. *Precipitation of gliadin from* 40 *cc. of an alcoholic solution.*

cc. *N*/50 HCl added	cc. *N*/50 NaOH added	Precipitate cc.	p_H
1·2	—	0·15	3·6
0·8	—	0·20	3·9
0·4	—	0·20	4·2
0·2	—	0·35	4·4
0	0	0·40	4·6
—	1·0	0·50	5·0
—	2·0	**0·55**	**5·2**
—	3·0	0·45	5·5
—	4·0	tr.	5·7
—	5·0	—	6·4

The point of maximum precipitation thus appears to be about p_H 5·2.

On theoretical grounds it appears to be somewhat unlikely that gliadin has an isoelectric point of p_H 9·3. Its content of arginine is low, lysine is practically absent and the proportion of glutamic acid present is very high (about 40 %). An isoelectric point of p_H 9·3 would make it resemble lysine (9·0) and arginine (10·48) which stand almost alone even among the amino acids of protein degradations in this respect. In addition to the fact that the greater part of the amino acids obtained on hydrolysis of gliadin have isoelectric points in acid media, it is known that the dipeptides are usually more acid than the amino acids from which they are obtained. Thus the p_H values of the isoelectric points of glycine, alanine and leucine are respectively 6·6, 6·7, 6·5 while those of glycyl-glycine, alanyl-glycine and leucyl-glycine are 5·5, 5·5 and 5·7 respectively. This suggests that the peptide linking tends to increase dissociation of $H^{\cdot}$ more than that of OH'.

Leucosin is the albumin present in wheat. There is less than 1 % in the endosperm, but about 5 % in the embryo [Osborne, 1909]. This protein is important as it is one of few vegetable proteins known which is physiologically active and not merely a storage form. It is best obtained in aqueous extracts of whole wheat meal, and is the only carefully studied vegetable protein which cannot easily be precipitated by acid. It is, however, coagulated on heating in fresh aqueous extract. The longer the extract stands the more acid it becomes and the more easily the protein is coagulated by heat [Osborne, 1909]. The only acid with which we could obtain a precipitate at room temperatures was nitric, and the precipitates were too small to be accurately measured. We therefore made use of the heat effect. Equal quantities of fresh aqueous extract were treated with varying proportions of acetic acid and then simultaneously heated to 47°. The precipitates varied in consistency and were therefore dried and weighed. Maximum precipitation occurred at p_H 4·5.

Table VI. *Precipitation of leucosin from* 20 *cc. of fresh extract.*

cc. *N*/10 acetic acid added	p_H	Precipitate in mg.	cc. *N*/10 acetic acid added	p_H	Precipitate in mg.
0	6·3	0	6·6	4·3	39
0·6	5·3	tr*	8·3	4·25	30
1·7	4·8	31	10	4·2	25
3·3	4·5	65	11·7	4·1	20
5·0	4·4	42			

* Solution becoming turbid.

4. *Potato* (Solanum tuberosum).

The principal protein extracted from potato is the globulin, tuberin, which can be obtained either in the expressed sap or in salt extractions. The isoelectric point of this protein was found to be at p_H 4·3–4·5 by Cohn, Gross, and Johnson [1919], a result we have confirmed—our estimations giving maximum precipitation at p_H 4·4–4·5. Using 10 % sodium chloride extractions of dried potato, the value was rather lower (p_H 4·2–4·4) and there was a secondary maximum of precipitation at p_H 3·2. Extraction of dried ground

potato with $N/100$ NaOH gave a solution from which the maximum precipitate was obtained at p_H 3·3, the precipitate giving protein reactions. There thus appears to be a second protein in potato, not obtained in quantity in the expressed sap, with an isoelectric point at p_H 3·2–3·3. It is possible that this may be a degradation product of tuberin since it is most apparent in tissue dried or treated with alkali.

Table VII. 10 % *NaCl extract of dry potato powder*—20 *cc. taken.*

cc. of N HCl added	Precipitated cc.	p_H	cc. of N HCl added	Precipitated cc.	p_H
0	0	5·4	0·8	1·1	3·7
0·2	0·8	4·6	1·0	1·0	3·4
0·3	1·4	4·4	1·2	**1·8**	**3·2**
0·4	**1·8**	**4·3**	1·4	1·2	2·7
0·6	1·5	3·9			

Yeast. Precipitation methods have already been utilised to determine the isoelectric points of small unicellular organisms [Eggarth and Bellows, 1922]. Yeast suspensions were found to precipitate most rapidly in the vicinity of p_H 3·0. The cells remained almost completely suspended in solutions of p_H 4·8–10·0. Maximum precipitation and the clearest supernatant liquids were obtained at p_H 3·3–3·5. Considerable precipitation occurs when the p_H value is lower than 4·6 and there may be a secondary substance present with an isoelectric point in this region (cf. the bean globulins). The main isoelectric point of yeast appears to lie near p_H 3·3.

Table VIII. *Yeast suspended in tap water.*

No.		Series I p_H	Series I Precipitate in cc.		Series II p_H	Series II Precipitate in cc.	
1	Water + NaOH	10·0	0·3	Very turbid	10	0·3	Very turbid
2		9·0	0·3		9·5	0·3	
3		8·0	0·3		9·0	0·3	
4		7·3	0·3		7·5	0·3	
5		6·3	0·3				
6	Water	4·8	0·4		4·8	0·4	
7	Water + HCl	4·6	0·6	Turbid	4·4	0·7	Slightly turbid
8		3·3	1·0		3·7	0·7	
9		2·9	0·8	Clear	3·5	0·8	
10		2·4	0·7		3·3	0·9	Clear
11		2·0	0·9		3·1	0·9	
12		1·4	0·85	Turbid	3·0	0·8	
					2·8	0·8	

8 and 9 precipitating very rapidly on adding acid.
Series II: sodium carbonate used for the alkaline solutions and acetic acid for the acid.

Nitella. This alga possesses long cells and a minimum of wall tissue, and it is easily obtained in quantity. On grinding up the tissues in water or in 10 % salt solution and filtering through cotton wool a slightly greenish suspension is obtained. If this is acidified the suspended matter slowly precipitates, the maximum precipitation occurring at p_H 4·6 after 24 hours. The precipitate gives protein reactions and microscopical examination shows it to consist chiefly of extruded masses of protoplasm—with a few embedded

chloroplasts. About one-tenth of the maximum precipitate consists of free starch grains. These apparently settle equally rapidly in all the solutions and are practically all precipitated within two hours. *Nitella* protoplasm has therefore an isoelectric point near p_H 4·7.

Table IX. *Precipitation of protein from* 20 *cc. aqueous extract of* Nitella.

cc. of *N*/30 HCl added	Precipitate in 2 hrs. (cc.)	Precipitate in 24 hrs. (cc.)	p_H
0	tr.	0·4	6·0
0·2	0·2	0·4	5·7
0·4	0·2	0·4	5·4
0·7	0·2	1·5	4·9
1·0	0·3	**1·7**	**4·6**
1·5	0·6	0·9	4·3
2·0	0·4	0·5	4·0

Edestin. The isoelectric point of edestin has been determined by Rona and Michaelis [1910], using the precipitation method and phosphate buffer solutions, to be at p_H 6·9. Later, using acetate buffers Michaelis and Mendelssohn [1914] obtained a value of p_H 5·6. Hitchcock [1922] estimated the isoelectric point of edestin to be between p_H 5·5 and 6·0 on the results of precipitation, osmotic pressure and cataphoresis observations. The samples of edestin used by Hitchcock combined with phosphoric acid in solutions of less than p_H 5·0. The estimations have been repeated using disodium phosphate-citric acid buffers and also using hydrochloric acid as the precipitating agent. For the specimens of edestin used (commercial and purified extracts from hemp seeds) maximum precipitation was obtained at lower p_H values than 5·6 (*e.g.* p_H 5·3, phosphate buffers) but the value obtained seems to depend on the previous treatment and purity of the product. No evidence has been obtained in confirmation of the value given by Rona and Michaelis of p_H 6·9.

Conclusion.

A representative series of plant proteins have thus been examined and the results indicate that the isoelectric points of plant proteins are usually about p_H 4·5 or less often about p_H 3·3. Edestin is the only exception to this though possibly gliadin may resemble edestin. The few additional records we have been able to find show the same features, the results being summarised in Table X.

Table X. *Summary of Isoelectric Points* (p_H *values*).

	p_H		p_H	
Vicilin	3·4	Edestin	5·6	Michaelis and Mendelssohn
Legumin	4·4–4·6		5·5–6·0	Hitchcock
Legumelin	4·2	Carrot globulin	4·1–4·4	Cohn, Gross and Johnson
Glutenin	4·4–4·5	Nucleoprotein (pancreas)	3·5	McClendon
Gliadin	3·5–5·5 (? 5·2)	Nucleoprotein (paratyphus bacilli)	4·0	—
Leucosin	4·5	Bacterium coli	3·2	Eggarth and Bellows
Tuberin	4·4	Bacillus of rabbit septicaemia	4·2	Northrop and Kruif [1922]
Potato	3·2	B. typhosum	3·5	—
Yeast	3·1–3·3	—	—	—
Nitella	4·6–4·7	—	—	—

We do not wish to discuss at present the theoretical questions suggested by this table, but only to draw attention to one feature of interest. Since the cell sap of parenchymatous tissue in plants is usually slightly acid (p_H 5·5–6·5) it is clear that the principal proteins are normally on the alkaline side of their isoelectric points and hence behave as anions. There are exceptions to this generalisation, notably in unripe fruits, and occasionally elsewhere. If, however, our results are as representative as we believe them to be, an important property of the protein constituents of plant protoplasm is indicated, which throws considerable light upon many questions of permeability and of metabolism.

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XLV. THE SUPPOSED INFLUENCE OF IRRADIATED AIR ON GROWTH.

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(*Received January 18th, 1924.*)

In dealing with such a subject as the discomfort felt in close atmospheres, people are generally inclined to turn away from simple physical causes and find an explanation of their ill feelings in some mysterious quality such as the presence of an organic poison exhaled by people, or the ionisation of exhaled air. Physiological research while disproving the presence of any organic poison has shown that the influence of temperature, humidity, and movement of air on loss of body heat and moisture is very great, and that comfort can be secured by ventilation so arranged as to give an adequate cooling and evaporative power to the air. This can be measured by the dry and wet bulb kata-thermometer.

While any evidence as to the influence on comfort of ionisation of the air has been wanting Miss Hume and Miss Smith [1923] have recently published a paper in which they claimed that short daily exposures to air, which had been irradiated by ultra-violet rays, stimulated to some extent the growth of young rats fed on a diet deficient in vitamin A.

The evidence adduced in their paper is not strong when considered on a statistical basis, but the novelty of their claim excited general attention and, while ignorant writers in the Daily Press went so far as to say herein lay the explanation of the ill effect of tropical climates on white men, an engineer interested in this matter claimed no less that in some ionisation of air lay the explanation of the discomfort felt in the House of Commons, a discomfort which Leonard Hill in 1914 had shown to be due to the fact that the feet of the members were cooled by the current of air impelled through the perforated floor while their heads were made hot by immersion in a relatively stagnant warm atmosphere.

It may be mentioned here also that Kestner [1923] has claimed that anaemia experimentally produced in dogs is influenced beneficially by inhalation of gases coming from an arc lamp—he has even affirmed that the active gas is nitrous oxide[1]!

In 1923 we gave the results of some experiments on growth of rats which were negative both as regards traces of ozone, oxides of nitrogen and tobacco smoke and irradiated air [1923]. We have carried further the experiments on irradiated air, detailed below, and have failed to find any positive results in these.

[1] Kestner moreover has asserted that the oppressiveness of a Sirocco wind is due to a fall of blood pressure produced by the presence of nitrous oxide in the air!

Experimental Methods.

(1) *Lamps.* We have made use of two different quartz lamps: (i) An automatic lighting lamp made by Kelvin, Bottomley and Baird which takes 2 amps. at 200 volts. The amount of ozone produced is small, but the lamp is very active in other respects. (ii) A lamp with a six-inch tube made by the Hewettic Electric Co. This takes 3·5 amps. at 200 volts and its effective radiating surface was about six times that of the K.B.B. lamp. It was very active as an ozone producer. Both lamps were new when used for these experiments.

(2) *Animals.* Rats were used in all cases. In some experiments the young rats were taken directly from the Institute stock without regard to parentage; in other experiments pregnant females were taken from stock and fed on a diet rich in vitamin A, during the later stage of pregnancy and throughout the period of lactation. The rats commenced their irradiation treatment the day after they were put on the diet deficient in vitamin A.

The rats were kept in the laboratory singly in large glass jars, 8 litres in capacity, with wire covers and were bedded on sawdust. In one experiment cotton-wool replaced the sawdust as bedding.

(3) *Diet.* The diet used had the following composition:

		Salt mixture	
Caseinogen	18 parts	NaCl	5·2
Rice starch	52 ,,	$MgSO_4$	16·4
Hydrogenated cotton seed oil	15 ,,	NaH_2PO_4	10·4
Marmite	5 ,,	K_2HPO_4	28·6
Lemon juice	5 ,,	$CaH_4(PO_4)_2$	16·2
Salt mixture	5 ,,	Ferric citrate	5·9
		Ca lactate	18·3

The caseinogen was the "edible casein" of commerce and was purified by (1) repeated extraction with boiling alcohol followed by extraction with ether, (2) heating in shallow zinc dishes to 102–105° for 72 hours, (3) a combination of both these methods.

The caseinogen, starch and salt mixture were first thoroughly mixed in a large mortar in a dry condition, then the melted fat was well kneaded in and finally the whole mixed into a stiff paste with the marmite, lemon juice and water. The diet was prepared fresh daily, and the rats were fed *ad libitum*. The lemon juice was expressed from the pulp of fresh lemons and was not decitrated or treated in any way.

The animals were weighed twice weekly in a tared vessel on a laboratory balance sensitive to 0·2 g.

Exp. 1. *See Chart* 1. Six young white rats about 50 g. each taken from stock, and divided into three groups of two each. The first pair were controls and received the diet deficient in vitamin A only; the second pair also received

10 minutes irradiated air daily, and the third pair 20 minutes irradiated air daily.

The irradiation was performed as follows: the rat was taken out of its jar and the sawdust was removed and the jar cleaned and taken to an adjoining room where the lamp was running; the jar was placed in the vertical position right under the lamp, the mouth of the jar being within two inches of the quartz burner. The jar was left there for a period of ten minutes and then quickly closed with a tight fitting glass lid and rapidly removed to the next room. Here the lid being opened slightly, the rat was quickly introduced and the lid then closed again. The rat remained in the closed jar for ten minutes after which the lid was taken off and the bedding and food replaced. This operation was repeated daily. In the case of the 20 minutes' exposure experiment, this was done twice a day.

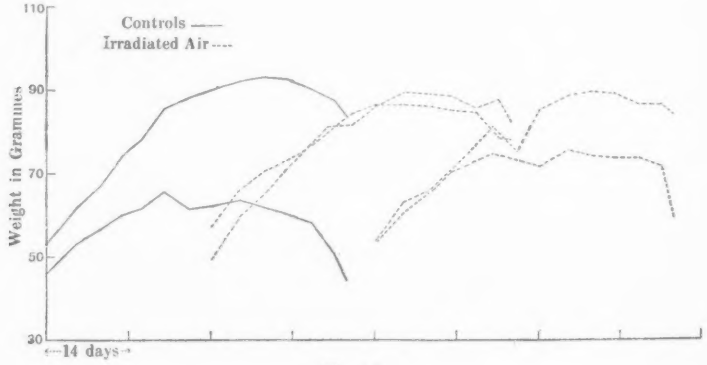

Chart 1.

This experiment lasted seven weeks and the lamp used was the Kelvin, Bottomley and Baird.

Reference to Chart 1 shows that the irradiated air was without effect on the growth of the rats. The variations of the curves were well within normal limits.

Exp. 2. *Chart* 2. Four young stock rats about 50 g. weight; one used as a control; one given irradiated air; one directly exposed to the rays of the lamp for a period of five minutes daily at a distance of two feet from the lamp—after two weeks of this treatment the exposure was increased to 10 minutes daily and finally for the last six days the distance from the lamp was shortened to 12 inches. To the diet of the fourth rat 3 % of cod-liver oil was added in place of an equivalent amount of the cotton seed oil. The same technique was used for the irradiated air as described in Exp. 1 except that the jar used had a capacity of 13 litres. The period was 10 minutes daily.

The experiment lasted five weeks and was then discontinued. Reference to the growth curve shows several features of interest. First, the powerful

effect of the cod-liver oil. This rat reached a weight of 175 g. in five weeks. Secondly, the very poor growth of the irradiated air rat compared to the control; in fact, the control rat grew so well that the caseinogen was suspected of being insufficiently purified, and was subjected to a further process of extraction or oxidation without, however, any effect on the growth of the controls. These rats must have had a good initial store of vitamin. The absence is noteworthy of any effect on growth of the direct rays of the lamp —as compared with the control—but we lay no stress on this, as both control and rayed rats were still growing when the experiment was stopped. If by chance we had used the irradiated air rat as a control and *vice versa*, a result in favour of the latter would have been apparent. Individual differences must be ruled out by a sufficient number of experiments.

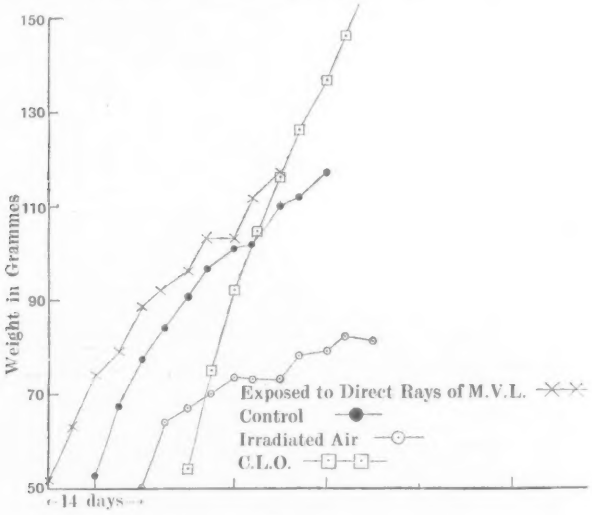

Chart 2.

Exp. 3. *Chart* 3. In this experiment, four rats from the same litter were used. During lactation the mother was fed on a diet of bread, milk, greenstuff, egg-yolk, and cod-liver oil. The irradiation was carried out by a somewhat different method in this experiment. A large bell jar about 3 litres capacity was used. It had two openings at the top one inch in diameter and could be closed at the bottom by a ground glass plate. After placing the rat in the jar and sealing the bottom with the glass plate the air from the vicinity of the burning lamp was sucked through the jar continuously by means of a filter pump. A short piece of india-rubber tubing (1 inch in internal diameter) was set from within an inch of the quartz lamp to the inlet of the jar. The outlet was closed with a rubber cork containing a tube running to the bottom of the jar and attached to a water pump. The actual length of the other tube

between lamp and jar was 12 inches. The jar was shaded from the light of the lamp.

By this means a continuous stream of irradiated air was sucked through the jar traversing only 12 inches of wide rubber tubing before entering the jar. One rat was kept in this apparatus 15 minutes a day and another rat was kept there 45 minutes a day. During the rest of the time the rats lived in their usual glass jars. A third rat was used as a control and the fourth was exposed to the direct rays of the lamp for a period of ten minutes daily at a distance of two feet; on the 21st day of the experiment the distance was shortened to one foot, and exposures were continued at this distance for the remainder of the experiment.

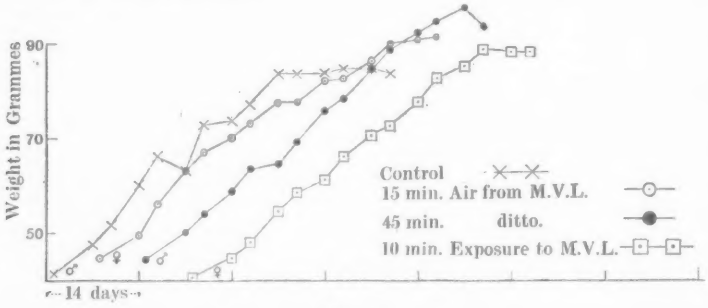

Chart 3.

Reference to the growth curve, Chart 3, shows that all four rats fall within the normal limits of a group of rats fed on a diet deficient in vitamin A. The irradiated air rats again fail to show the great increase in growth claimed by Hume and Smith.

As the fact that the rat receiving the direct rays of the lamp failed to grow better than the others was surprising, it was decided by one of us (T.A.W.) to see if the Ca and P content differed in any way from that of the other rats. The four rats were killed and the viscera removed and the carcases were then ashed and a phosphorus determination made. The results are as follows:

Control rat	0·469 g. P or 55·6 mg.	per g. of rat
15 minutes R.A.	0·487 g. P or 53·5	,, ,,
45 minutes R.A.	0·513 g. P or 55·1	,, ,,
M.V.L.	0·595 g. P or 67·6	,, ,,

It will be seen that the rat subjected to the direct rays of the lamp contained a significantly greater amount of P than the other rats. The direct rays of the lamp had excited their well-known antirachitic effect, without any significant effect on the growth of the animal.

It has been shown by Korenchevsky [1921], and Goldblatt [1923] that the bones of rats fed on the $-A$ diet used here do not have a normal calcium phosphate content. The diet is rather low in calcium.

It should be stated that none of the rats showed any macroscopic signs of rickets—the predominating feature in autopsy being the almost complete absence of subcutaneous fat.

The K.B.B. lamp was used in this experiment and the rats bedded in cotton wool.

Exp. 4. *Chart* 4. In this experiment six rats of a litter of seven were used. The mother was fed on diet rich in vitamins three days before the birth of the litter and this rich diet was continued until the experiment was started.

The litter was divided into three groups of two each. One group was used as controls, one for treatment with irradiated air and one for treatment by the direct rays of the lamp.

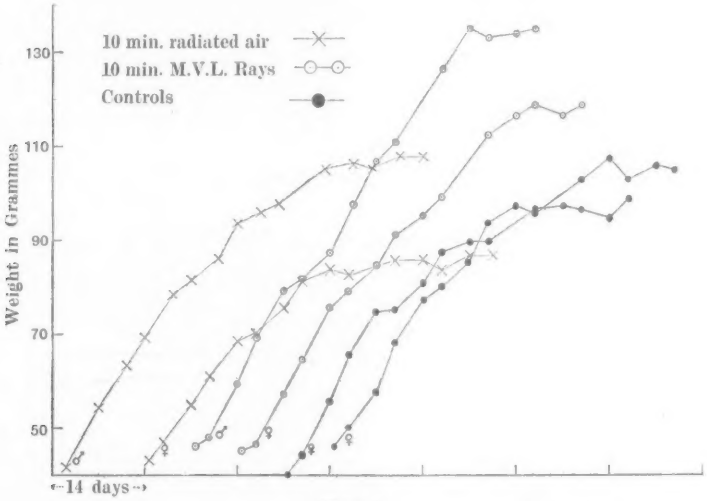

Chart 4.

The irradiated air treatment was carried out as in Exp. 2, a 13 litre jar being used, the period being ten minutes daily. The direct raying of the rats was 15 minutes daily at a distance of two feet from the lamp. The lamp used was the large lamp made by the Hewettic Electric Co. The rats used varied in colour as follows: irradiated air treatment; one white and one black and white; controls; one black and white, and one sable; direct raying with M.V.L.; one white and one dark brown; this brown rat very much resembled a wild rat except for a few white patches on its belly.

The experiment lasted seven weeks and the results are shown in Chart 4. The irradiated air rats again show no growth response but the rats exposed directly to the rays from the lamp appear to show a distinct growth response. This is more noticeable in the case of the brown rat. As it is well known

that this type of rat grows better than the albino rat, we cannot assert that this deeply pigmented rat shows better growth in response to radiation than the albino rat.

We are making further investigations into this question of pigmentation and light effect.

Discussion.

In no case have we been able to confirm the results of Hume and Smith regarding the growth-promoting effects of irradiated air and we have come to the definite conclusion that it has no effect on growth, nor do we see any possible *a priori* reason why it should have an effect on growth.

A daily exposure of 15 minutes to the rays of a 3·5 ampere lamp at a distance of two feet appears to have some stimulating effect on the growth of young rats on a diet deficient in vitamin A, but a daily exposure of ten minutes to a 2 ampere lamp apparently has no effect on growth although it exercises a considerable effect on the calcium and phosphorus metabolism. Evidently the ultra-violet rays do not stimulate growth by preventing rickets as suggested by Steenbock and Nelson [1923]. The radiant energy necessary to promote growth appears to be other than that required to cure rickets.

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XLVI. THE PART PLAYED BY IRON AND FAT IN THE RECOVERY OF RATS FROM CHRONIC EXPERIMENTAL ANAEMIA.

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(Received January 22nd, 1924.)

Iron.

The problem of the degree to which inorganic iron was absorbed from the alimentary canal was one which attracted a great deal of attention in the years between 1885–1900, a fact which was largely due to the work of Bunge. Bunge's view was that inorganic iron was not absorbed, though the administration of such might indirectly aid absorption of organic iron by uniting with the sulphuretted hydrogen in the alimentary canal, thus screening the organic iron. In later years Bunge seemed to give up this view.

Nevertheless there still seemed to be some point in considering in the light of new knowledge the factors which influence the absorption of iron, and other experiments which were recently performed and which have already been described in this Journal [Scott, 1923; Scott and Barcroft, 1924] gave me the opportunity of doing so and lead up rather naturally to it.

It has been stated by Whipple [1918] that in his experiments administration of inorganic iron salts had no effect upon blood regeneration in simple anaemia in dogs. This conclusion is at variance with clinical experience, with earlier experimental work summarised by Sherman [1907], and with the recent experimental results of Schmidt [1912] on mice, Brinchmann [1921] on guinea-pigs, and Hirasawa [1923] on rabbits. In the case of rats made anaemic by breeding on diets of bread and milk [Scott, 1923], the results in Table I demonstrate that inorganic iron has the effect of hastening the cure of the anaemia. When, from the age of 8 weeks, ferrous chloride is given at the rate of 20 mg. per rat per day a cure is reached by the age of 16 weeks. The result shown has been obtained repeatedly and without exception, and the contrast between the blood of the treated animals and the controls is very striking even to the naked eye. The cure is slightly less rapid than that which occurs when green leaves are added to the bread and milk but anticipates the cure which occurs naturally at the age of about six months when the rats are kept on bread and milk alone [Scott, 1923]. Thus the results of the last three workers cited above are confirmed in rats but one has yet to bring the

results of Whipple's experiments into the general scheme. They appear to indicate the contrary conclusion; but a little consideration will show (1) that administration of inorganic iron cannot be expected to have much effect unless the diet is already poor in iron and (2) that the methods adopted by Whipple for producing anaemia may not have been sufficiently drastic seriously to deplete the reserve stores of iron in the tissues; Whipple's animals always had these reserves to draw upon and consequently the administration of iron to them was a work of supererogation.

Table I. *Showing effect of inorganic iron on blood regeneration in anaemic rats at 16 weeks of age. Diet—white bread and whole milk; 20 mg. ferrous chloride added per rat per day.*

	Haemoglobin %	Red blood count in millions	Colour index (human = 1)
	103	9·98	·52
	101	9·46	·53
	95	8·80	·54
	98	9·12	·54
	107	10·02	·53
	98	9·62	·51
	98	8·74	·56
	96	8·15	·59
	94	9·47	·50
Average	99	9·26	·53
Average of controls at same age on bread and milk without iron			
	55	10·03	·28

Fat.

There seems to have long been an idea that fat is connected in some way with blood formation. References are given by Stockman [1895]. It has been shown by Geiling and Green [1921] and Jencks [1922] that in rats regeneration of red blood corpuscles after a single or double haemorrhage, especially the latter, occurs more rapidly if the animals be on a diet rich in vitamin A. This observation has been confirmed in the case of experimental anaemia in rats, for green leaves and (unpublished experiments) bird seed accelerate the natural cure which occurs on a bread and milk diet at about six months [Scott, 1923]. Hamburger [1921, 1922] has brought forward presumptive evidence that fat and lecithin are necessary for blood regeneration in rabbits. The lipaemia that follows on haemorrhage in these animals, possibly a prelude or accompaniment of blood regeneration, is of course well known [Bloor and Macpherson, 1917; Horiuchi, 1920]. Hamburger's experiments however lend themselves equally well to the interpretation that a supply of vitamin A is essential for blood regeneration. Further evidence has been brought forward that in rats the supply of fat in the diet is comparatively unimportant, the important thing being the supply of fat-soluble A [Drummond, 1920, 1921; Osborne and Mendel, 1920]. Geiling and Green [1921] find that in rats on a fat-deficient diet regeneration after double haemorrhage occurs in normal time. The same

criticism may be directed against a too rigid interpretation of this work as was levelled above in the case of Whipple's work; but the evidence cited so far would lead one to suppose that it is not fat but vitamin A that is essential in blood regeneration. This constitutes the evidence one has to build up and it is somewhat contradictory.

Large numbers of rats with a sub-chronic anaemia could be obtained and it seemed that the following inquiries might aid in further elucidating the matter:

1. Does the fat of milk play a part in the recovery of rats from the sub-chronic experimental anaemia described?

2. Can equally good recovery be obtained if a hydrogenated fat be substituted for milk fat?

Anaemic rats which had been fed on bread and whole milk were placed at 12 weeks of age on a diet of bread and skimmed milk plus 20 mg. of ferrous chloride and 2 g. palm kernel oil per rat per day. Table II demonstrates the findings at 20 weeks of age.

Table II. *Showing effect of bread and skimmed milk plus* 20 *mg. ferrous chloride and* 2 *g. palm kernel oil per rat per day on blood regeneration in anaemic rats at* 20 *weeks of age.*

Haemoglobin %	Red blood count in millions	Colour index (human = 1)
61	10·03	·30
61	9·67	·31
58	9·43	·31
68	10·32	·33
58	9·24	·31
61	11·21	·27
63	9·82	·32
51	8·96	·28
Average 60	9·84	·30
Average of controls on bread and whole milk and ferrous chloride		
98	8·68	·56

Conclusion.

Iron. The diet on which this anaemia develops in rats is a comparatively iron-poor diet; on this diet natural cure occurs slowly as the animals get older. Since this is so and since the sole addition of iron to the diet results in a hastening of the natural process of cure it may be concluded that the anaemia is due to deficiency of iron in the diet. It is therefore another deficiency disease.

Fat. In the case of rats fed on bread and milk with or without the addition of iron the milk fat or something associated with it is necessary for blood regeneration, but the evidence is insufficient to decide whether its absence or the diminished supply of vitamin A leads to a state of chronic under-nutrition, whether its function lies in facilitating absorption of iron [Macallum, 1894] or whether it is necessary for the functioning of the haemopoietic tissues or

directly for the construction of the haemoglobin molecule. One must admit the possibility of the first explanation for the reasons that on this diet (1) they show signs of under-nutrition in their rough coats, and (2) they show dilated stomachs, presumably because they have to take a large bulk of food to secure a sufficiency of calories. One must therefore be cautious not to claim too much from these experiments, but they do demonstrate the broad (and self-evident) fact that iron will not effect a cure if the diet be otherwise inadequate.

It is of interest that this result should be obtained, for it provides an experimental analogy to the clinical experience that anaemias of chlorotic type are sometimes met with which are refractory to treatment with iron alone [Stockman, 1895]. If one may reason from the analogy of the rats a possible explanation is that administration of iron is without avail where the diet is otherwise inadequate, particularly in the supply of vitamin A. It has been shown above that administration of inorganic iron, though efficacious, is in no way superior to feeding natural foods containing iron, and the latter have the great advantage of also containing vitamin A, which would appear to play a part in blood regeneration. The use of suitable natural foods is thus preferable to the administration of iron in the treatment of anaemia.

Summary.

1. The anaemia discussed is a deficiency disease and is due to lack of iron in organic or inorganic form.

2. Administration of iron is without avail for its cure where the diet is otherwise inadequate, particularly in respect of the only natural fat which has been tested—namely that of milk. Vitamin-free fat (palm kernel oil) does not supply the deficiency.

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XLVII. A QUANTITATIVE STUDY OF SUCCINIC ACID IN MUSCLE. I.

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Introduction.

That muscle-tissue contains a very powerful enzyme for the oxidation of succinic acid has been known since the work of Thunberg [1909], and specially of Batelli and Stern [1911], and this fact inevitably suggests that the acid may have an important rôle in muscle respiration. In the present paper is described an attempt to discover, by following quantitatively the changing concentrations of succinic acid under various conditions, the dynamic part played by the acid in the muscle.

Einbeck [1913, 1914] showed that perfectly fresh muscle tissue contains succinic acid; he thus disproved the older view that the succinic acid found in muscle extracts was always a product of putrefaction [Wolff, 1904, and many others], or a product (in the course of analysis) of a complex substance, carno-phosphoric acid [Siegfried, 1903]. Einbeck succeeded in preparing 0·112 g. of pure succinic acid from a kilogram of beef two hours after the death of the animal, but his method was unsuitable for quantitative work; he showed an increase in the succinic acid content of the meat, but only after long periods of storage (two and six weeks at 2°), when attack by moulds and bacteria had begun.

In the present investigation, absolutely fresh muscle was always (except in the case of the frog) found to yield a small quantity of succinic acid; this yield could be increased by incubating the minced muscle in suitable buffer solutions, and the effect on this yield of various factors (such as changing hydrogen ion concentration, added sugar, amino-acids, toluene, tissue extracts, etc.) has been studied. Beef which had been exposed for sale was found to contain no succinic acid, or not more than 0·5 mg. per 100 g. This must mean that the power of the muscle to oxidise the acid persists longer than the power to produce it; so it is not surprising that the older investigators, such as Wolff, whose freshest samples were one day old, should have entirely missed the presence of succinic acid before putrefactive changes set in.

Unfortunately, the increase in the succinic acid yield found, though relatively large (in most cases 4–6 fold) is absolutely very small, and this fact has made it impossible to obtain so far any direct evidence as to the precursor of the acid.

Method of Estimation.

The essential part of the method employed was the volumetric estimation of silver in the silver succinate precipitate in the muscle extract after the removal of all other interfering muscle constituents.

The minced muscle was first ground with sand and 50 % alcohol (according to Batelli and Stern [1911] this concentration of alcohol is sufficient to inhibit the action of the succinoxydase) and left to stand overnight. The mixture was strained through muslin, and the residue extracted twice more with 50 % alcohol. After filtering through paper, the extract was evaporated on a boiling water-bath nearly to dryness in a large dish. The protein which separated out was filtered off, and washed with boiling water. The filtrate was then evaporated down to dryness in a small dish.

The brown fatty residue was rubbed up very thoroughly with successive small quantities of cold saturated ammonium sulphate solution, and filtered in a special apparatus. This consisted of a 10 cc. cylindrical funnel, fitted with a capillary tap. At the top of the capillary, just above the tap, was placed a small flat piece of porous pot, and above this a pad of asbestos, about 2 mm. thick, was made. The capillary tube of the funnel passed through a rubber stopper, into an adapter, which communicated by a side-tube with the filter-pump, and which in turn fitted into the rubber stopper of a 100 cc. measuring cylinder. Not more than 20 cc. of saturated ammonium sulphate solution were used; the residue which collected on the pad was well stirred to ensure complete washing during the latter part of the filtration. This treatment with ammonium sulphate solution, as described by Meyerhof [1921] in his modification of Parnas' method of lactic acid estimation [1915], removes all the remaining protein and fat, and a beautifully clear yellow or brown filtrate is obtained; the high salt concentration also has a favourable effect on the partition coefficient in the ether extraction stage.

One-tenth of the volume of concentrated sulphuric acid and a little solid ammonium sulphate were added, and the solution was extracted with thrice its volume of ether five times, by shaking by hand. The ether was each time siphoned off through a dry filter paper into a flask containing about 10 cc. of water. The ether was distilled off, and the aqueous residue cooled, filtered, and made up to about 100 cc.

To this were added 3 cc. of concentrated sulphuric acid, and the flask was heated on a boiling water-bath; $N/10$ potassium permanganate was run in from a burette until a permanent brown precipitate was obtained. This permanganate titration is primarily to remove lactic acid, and acts as a fairly dependable rough indication of the amount of lactic acid present; sugar has

been removed in the ether extraction, and the only other substances to be oxidised are possible traces of fumaric, malic, and hydroxyglutaric acids, etc.

The oxidised solution was evaporated down to about 10 cc.; it was saturated with ammonium sulphate, and again extracted with pure ether five times in the same manner as before. This second extraction stage is necessary to get rid of the large amounts of sulphuric acid and manganese salts present. After removal of the ether, the faintly acid aqueous solution was made faintly alkaline with very dilute potassium hydroxide solution (to decompose any ammonium salts, which interfere with the precipitation of the silver succinate) and evaporated down to 10 cc. It was then made faintly acid again with dilute nitric acid, and 1 cc. of 10 % silver nitrate was added; the solution was neutralised with dilute ammonia and the silver succinate suspension warmed on a water-bath to coagulate the precipitate and then allowed to stand for 3–12 hours. It is important to precipitate in acid solution, neutralising afterwards, as it is often difficult to *start* the precipitation in neutral solution.

The precipitate was filtered off into a Gooch crucible, and washed four times with very dilute ammonium nitrate solution. It is very important that a standard method of washing should be used for all estimations. Silver succinate is appreciably soluble in cold water (17·6 mg. per 100 cc. water at 18°) and it was found, on titrating the washings with potassium thiocyanate, that silver-free filtrates could not be obtained even after eight to ten washings; but by the fourth washing the silver content had become constant at a very small amount. The silver precipitate was dissolved in hot nitric acid (about 1 : 4), the solution cooled and titrated against $N/100$ potassium thiocyanate solution, using iron alum as an indicator.

The method was tested by adding small known amounts of succinic acid to muscle, and comparing the succinic acid content of this and of untreated muscle. The beef used in these experiments had been exposed for sale and contained only traces of succinic acid; the values given in the following table were obtained by subtracting these small amounts from the values found for the treated muscle.

Succinic acid added mg.	Found	% recovered	No. of ether extractions	
			First stage	Second stage
5·0	5·1	102	5	4
4·8	4·3	90	6	6
5·2	4·9	94	6	6
5·2	4·7	92	4	6
4·5	4·2	95	6	6
4·5	4·4	97	8	8
4·5	4·5	100	6	6
4·5	4·1	91	6	6
4·5	4·0	90	6	6

These examples of results show that on an average 95 % of the succinic acid present in the muscle extract is accounted for, with an error of ± 5 %.

In order to test the purity of the silver succinate obtained in the final stage, the silver in it was estimated; the free acid was also prepared (without recrystallisation), and its melting point taken.

850 g. of beef, fresh from the slaughter-house, were treated exactly as described, and the solution, ready for precipitation, was divided into two parts.

(*a*) The silver salt was precipitated; it was carefully washed by decantation, then filtered through a Gooch crucible, and washed further. The washings were free from sulphate. The crucible was then dried at 110°–115° to constant weight. The precipitate was dissolved in nitric acid and the silver estimated with potassium thiocyanate. Percentage found = 62·1. Calculated percentage = 65·0.

(*b*) The silver salt was precipitated and washed in a Gooch crucible. It was then suspended, together with the asbestos, in water and H_2S was passed. The liquid was filtered and the dark brown filtrate was evaporated down to small bulk to coagulate the colloidal silver sulphide. The evaporation and filtration were continued alternately until, on evaporating completely to dryness, a residue was obtained, which was completely soluble in water. This was dried at 110°. M.P. 177·5°. Mixed M.P. (with Kahlbaum's succinic acid) 177·5°.

On another occasion, beef in which the maximum amount of succinic acid had been allowed to accumulate, was worked up. The melting point of the free acid was 179°; the mixed melting point was also 179°. The percentage of silver in the silver salt was found to be 63·9.

It is thus seen that the silver salt obtained is silver succinate in a state of very fair purity, and also that the added succinic acid can be quantitatively recovered. There is, however, one reservation which should be borne in mind with regard to the trustworthiness of the results. It is possible that under certain conditions, *e.g.* incubation in phosphate solution, traces might be formed of some unstable substances (of which the most likely seems to be α-hydroxyglutaric acid) which in the course of the method of estimation might be converted into succinic acid. Thus, suppose that α-hydroxyglutaric acid was formed in the muscle, it is possible that this would yield succinic acid on oxidation with permanganate; this is a difficult point to investigate, but work is being carried out on the subject.

Succinic acid formation in minced muscle.

The method adopted in the hope of encouraging as great an accumulation of the acid as possible was the suspension of the minced muscle in an approximately isotonic buffer solution (Na_2HPO_4) at an alkaline p_H. To prevent oxidation of the acid formed, either nitrogen was bubbled through the mixture or potassium cyanide was added. In some cases the nitrogen was carefully freed from oxygen, and in other cases taken straight from the cylinder; similarly, the concentration of potassium cyanide was varied within

fairly wide limits, but these changes had no detectable effect on the yields of succinic acid.

Details of the experiments with muscle from different animals are given below.

(A) *Beef. Exp.* 1. The muscle was removed from the neck of the bullock immediately after death and brought to the laboratory in a pan surrounded by ice and salt. The muscle was quickly dissected out, and minced into a cooled dish, where it was well mixed. Two lots of 100 g. each were weighed out, and placed in two flasks containing 175 cc. of 1·4 % Na_2HPO_4 solution (p_H 8·4). To one flask 5 cc. of $N/10$ KCN solution were added (the concentration of KCN thus being about 1 in 5000), the other was connected to a nitrogen cylinder, and a slow stream was kept bubbling. The flasks were placed in a thermostat (36–38°) for 3·5 hours. At the end of the time 300 cc. of 97 % alcohol were added to each. As soon as the samples in the thermostat were ready, 50 g. of the muscle were extracted at once with 50 % alcohol.

	cc. of $N/10$ permanganate required %	Succinic acid in mg. %
Initial	118	5·6
After 3·5 hrs. KCN	260	34·0
" " nitrogen	270	30·0

The working up of the samples was carried out exactly as described, except that the 50 % alcoholic extract was neutralised before evaporation. Controls were performed to show that all phosphate and cyanide are removed in the course of the estimation and do not interfere with the silver precipitation. The precipitate obtained was always white on first coming down.

(B) *Dog. Exp.* 2. Muscle from the thigh was removed in one piece and cooled in a pan surrounded by the freezing mixture. When the temperature had fallen to about 20° some uninjured muscle was dissected out and further cooled to 7°. It was then minced and two lots of 50 g. each were weighed out. One lot was extracted at once, the other was suspended in 100 cc. of 1·4 % sodium phosphate solution.

	cc. of $N/10$ permanganate required %	Succinic acid in mg. %
Initial	73	2·0
After 3·5 hrs. at 33° in nitrogen	204	24·3

(C) *Sheep. Exp.* 3. The mutton was obtained about half-an-hour after the death of the animal; the lactic acid maximum, as indicated by the permanganate titration had already been reached before the experiment began, but evidence of succinic acid formation was obtained.

	cc. of $N/10$ permanganate required %	Succinic acid in mg. %
Initial	138	4·9
After 3·5 hrs. at 37° in phosphate at p_H 7·0	142	14·7
Ditto, in Tyrode's solution	118	11·0

(D) *Frog.* The results obtained here were surprising as at first no succinic acid could be detected either in the fresh or in the incubated muscle.

Exp. 4. July. The hind-limbs were cooled in ice; the muscle was dissected without injury, and extracted at once with ice-cold 50 % alcohol. Two samples were worked up and no succinic acid was found in either.

Exp. 5. November. The hind-limbs of 15 frogs were put into chloroform rigor (in an atmosphere of nitrogen) in order to see whether the increase of lactic acid obtained in this way was accompanied by an increase in succinic acid. The muscles from 15 control frogs were extracted at once with 97 % ice-cold alcohol.

	cc. of $N/10$ permanganate required %	Succinic acid in mg. %
Initial	8	0·7
$CHCl_3$ rigor	100	1·4

Exp. 6. March. Two lots of 50 g. of muscle, suspended in 100 cc. of phosphate solution at p_H 7·0, were kept at 37–40° for 3·5 hours, and KCN added to make the solution about 1 in 5000. The control was extracted at once with 97 % alcohol.

	cc. of $N/10$ permanganate required %	Succinic acid in mg. %
Initial	20	0·0
After 3½ hrs.	290	0·0
„	280	0·0

Exp. 7. December. In view of Laquer's work [1921] showing that the formation of lactic acid by muscle from added glucose does not take place at 45° while it does at 30°, it was thought that the high temperature might have had a deleterious effect on the factors concerned in succinic acid formation. A suspension of muscle in buffer solution (1·4 % phosphate) was therefore kept for 24 hours at room temperature (KCN about 1 in 5000) with the result that about 5 mg. % of succinic acid were found. Unfortunately no frogs were available for a control.

(E) *Rabbit.* When the mixed muscle of the whole animal was used, the succinic acid formation was found to be very small, though quite definite. 1·4 % phosphate solution was used and KCN as before.

Exp. 8.

	cc. of $N/10$ permanganate required %	Succinic acid found in mg. %
Initial	183	0·0
	165	0·0
After 1·5 hrs. (33°)	180	4·0
3·25 hrs.	204	5·3
4·0	197	5·4
5·0	208	5·9

In another experiment under similar conditions 4·2 mg. % of succinic acid were found after two hours.

Succinic acid formation in red and white muscle.

In consideration of the fact that Batelli and Stern [1912] found considerable differences between the activity of the succinoxydase in the red and white muscles of the hen and the calf—the enzyme being much more active in the red muscle—it was thought worth while to test the two kinds of muscle in the rabbit separately for succinic acid formation.

Two rabbits were used in each experiment. It had been thought that the smallness of the amount of succinic acid formed might be due to an injurious effect of the very rapid lactic acid formation in the mixed muscle of the rabbit; and that if the lactic acid formation could be delayed by thorough cooling, the succinic acid formation might get a better chance. After the rabbits had been skinned and drawn, therefore, the carcases were placed in a pan surrounded by a freezing mixture for over an hour before the muscle was dissected off and minced. The reddest muscle was dissected out and minced separately. One sample of red muscle and one of white were extracted at once; other samples were incubated in phosphate solution ($p_H = 8{\cdot}4$) with KCN at a concentration of 1 in 1700.

Exp. 9.

		cc. of $N/10$ permanganate required %	Succinic acid found in mg. %
White.	Initial	165	0·0
	3·5 hrs.	250	3·7
Red.	Initial	150	2·9
	3·5 hrs.	210	9·4

Exp. 10.

White.	3·5 hrs.	180	4·2
	3·5	185	4·7
Red.	3·5	114	10·4
	3·5	140	8·8

It must be pointed out that the red muscle used was very tendinous and that much of it was of a light red colour; probably if a sufficient quantity of the really dark muscle could be obtained, a still higher figure for the succinic acid would be found. The permanganate titration figures show clearly the much slower formation of lactic acid in the red muscle, as was found by Fletcher [1912]. It is interesting to note that, in Exp. 10, the sample giving the higher lactic acid figure (and therefore probably containing more pale muscle) gives a lower figure for succinic acid. Even with the greater precautions in cooling, the lactic acid formation in the white muscle goes on much more rapidly than in the muscle from the ox and from the dog.

The effect of added sugar, amino-acids, and toluene.

(*a*) *Sugar.* The addition of glucose to incubating beef muscle increases the lactic acid formation to an extent quite outside experimental error, without affecting the production of succinic acid. The samples were suspended in 1·4 % phosphate, and incubated at 37° for 3·5 hours.

		cc. of $N/10$ permanganate required %	Succinic acid found in mg. %
Exp. 11.			
	No glucose	294	29·1
		295	33·8
	Glucose added	370	34·7
		371	30·8
Exp. 12.			
	No glucose	257	22·9
	Glucose added	326	23·9

(*b*) *Amino-acids. Exp.* 13. The amino-acids tried were glutaminic and aspartic; 0·2 g. of each acid (in neutral solution) was added to one sample of muscle, suspended in phosphate solution at p_H 8·4, in an atmosphere of nitrogen. The sample and a control were kept at 37° for 3·5 hours.

Control	15·6 mg. % succ. acid
Glutaminic and aspartic acids added	83·4 „ „

Thus a markedly increased yield of succinic acid resulted from the addition of these amino-acids.

(*c*) *Toluene.* The addition of toluene interferes seriously with succinic acid formation, though only in certain cases with that of lactic acid. In the following table this is shown. Each sample of 50 g. of muscle was suspended in 100 cc. of 1·4 % Na_2HPO_4 solution, 5 cc. of toluene and 1 g. of sugar were added to certain of the samples as pointed out in the table.

Exp. 14. Conditions of experiment				cc. of *N*/10 permanganate required %	Succinic acid found in mg. %
Initial				(*a*) 115	6·9
3 hrs.	KCN 1 : 4000.	No sugar.	Toluene	(*b*) 251	14·8
3	N_2	„	„	(*c*) 247	21·7
3	N_2	„	No toluene	(*d*) 257	22·9
18	KCN 1 : 1500.	„	Toluene	(*e*) 257	16·5
18	KCN 1 : 4000.	„	„	(*f*) —	15·6
3	KCN 1 : 4000.	Sugar	„	(*g*) 261	14·0
3	N_2	„	„	(*h*) 296	20·5
3	KCN 1 : 4000.	„	No toluene	(*i*) 326	23·9
Exp. 15.					
3	KCN 1 : 5000.	No sugar.	No toluene	(*j*) 294	29·1
3	KCN 1 : 5000.	Sugar.	„	(*k*) 370	34·7
3	KCN 1 : 5000.	„	Toluene	(*l*) 340	12·0

These figures show:

1. The inhibitory effect of toluene on succinic acid formation. Compare (*b*) and (*g*), also (*c*) and (*h*), (*e*) and (*f*), with (*d*) and (*i*). The much smaller effect in the cases of (*c*) and (*h*) is probably due to the carrying away of the toluene vapour by the stream of nitrogen.
2. The inhibitory effect of toluene on the formation of lactic acid from *added* glucose. Compare (*g*) and (*h*) with (*i*), and (*l*) with (*k*). On the other hand the non-effect of toluene on the formation of lactic acid from the carbohydrate of the muscle itself. For this compare (*b*), (*c*) and (*e*) with (*d*).
3. Further examples of the non-effect of added glucose on succinic acid production. Compare (*b*) and (*c*) with (*g*) and (*h*).

It is extremely improbable that the larger increase in succinic acid found in the absence of toluene is due to bacterial action. In the first place, there is quite as much chance for bacterial action in the case of the frog and rabbit muscle, where the succinic acid production is nil or very small; and, in the second place, the time relations of the formation, as described later, are against the probability of bacterial action.

EFFECT OF THE ADDITION OF VARIOUS PANCREAS PREPARATIONS.

It was thought possible that the smallness of the succinic acid production might be due to the presence of only a very limited amount of its immediate precursor, and that some internal secretion—normally brought in the circulation, but absent from excised muscle—might be necessary for the production of new supplies of the precursor. For example, it was suggested that succinic acid production might be an alternative course to lactic acid production in carbohydrate metabolism, and that the former acid might arise from the breakdown of some special form of sugar. Now it seems that the pancreas is concerned in the production of the special form of sugar found in the blood [Winter and Smith, 1923, 1, 2]; moreover, Winfield and Hopkins [1916] had shown that the addition of ground pancreas to chopped muscle caused a marked inhibition of lactic acid formation. Experiments were therefore performed to see whether the carbohydrate which, in the presence of the pancreas, had not been converted into lactic acid, might possibly have been turned into succinic acid. Instead of the ground pancreas having a stimulating effect, however, a well-marked inhibition of the succinic acid formation (approximately proportional in most cases to the lactic acid inhibition) was observed.

Exp. 16. As before, perfectly fresh beef was obtained. A rabbit's pancreas was used, and ground with sand in Tyrode's solution, the p_H of which was adjusted to 7·0 by adding dilute hydrochloric acid. In order to get as thorough mixing as possible of the pancreas preparation with the muscle before any considerable acid production could take place, 150 g. of the muscle were weighed out and pounded in a mortar with the ground pancreas; this mixture was then divided into three equal samples. Two samples were suspended each in 100 cc. of phosphate solution at p_H 5·8, and well mixed. Two control samples were each suspended in the same amount of phosphate solution. Finally the requisite amount of soda to bring the p_H of the solution to 7·0, not allowing for the acidity of the tissues, was added to each flask. Two flasks, one control, and one with pancreas preparation, were connected to a nitrogen cylinder and kept in an atmosphere of nitrogen; to the other two flasks KCN was added (1 in 4000). The third lot of 50 g. was suspended in Tyrode's solution at p_H 7·0, and a control in the same solution was also prepared. All were kept for 3·5 hours at 37°.

			cc. of $N/10$ permanganate required %		Succinic acid found in mg. %	
Phosphate.	N_2.	No pancreas extract	302	60 % inhib.	21·5	55 % inhib.
Phosphate.	N_2.	Pancreas extract	126		9·8	
Phosphate.	KCN.	No pancreas extract	294	57 % inhib.	18·0	80 % inhib.
Phosphate.	KCN.	Pancreas extract	128		3·5	
Tyrode.	N_2.	No pancreas extract	160	19 % inhib.	17·0	20 % inhib.
Tryode.	N_2.	Pancreas extract	130		13·0	

Exp. 17. This experiment was performed in July, and the effect, both on

lactic and succinic acid production, was much less. This was probably due to the greater difficulty in cooling the tissue.

	cc. of $N/10$ permanganate required %	Succinic acid found in mg. %
No pancreas extract	310	20·8
Pancreas extract	220 } 30 % inhib.	16·3 } 22 % inhib.

Exp. 18. Mutton was obtained from the slaughter-house, but about 30 mins. had elapsed between the killing of the animal and the cooling of the meat. The experiment was carried out just as for the beef, two samples being suspended in phosphate solution, and two in neutralised Tyrode's solution. It was found that the lactic acid maximum had already been reached at the beginning of the experiment, and no inhibition was seen, *i.e.* no resynthesis of lactic acid seems to have taken place; but succinic acid formation and inhibition are clearly seen.

		cc. of $N/10$ permanganate required %	Succinic acid found in mg. %
Initial		(*a*) 138	4·9
Phosphate.	No pancreas extract	(*b*) 142	14·7
Phosphate.	Pancreas extract	(*c*) 130	10·4
Tyrode.	No pancreas extract	(*d*) 118	11·0
Tyrode.	Pancreas extract	(*e*) 122	8·2

Effect of absence of buffer. In this experiment the effect of the absence of the buffer, and the consequent rise in hydrogen ion concentration of the medium is seen in the smaller yield of succinic acid in (*d*) and (*e*).

Other pancreas preparations were tried in order to see whether the succinic acid production was always affected in a similar way to that of lactic acid.

Exp. 19. Commercial pancreatin was used, 0·4 g. being added to each of samples (*c*) and (*d*). The pancreatin used for sample (*e*) was first digested with alkali in order to destroy trypsin: 1 g. was mixed with 50 cc. of 1 % Na_2CO_3 solution and left for 20 hours at 37° with toluene. The neutralised solution was found to be trypsin-free, for there was no digestion of casein after 48 hours; the toluene was removed by warming to 40°. 20 cc. were used for sample (*e*). The experiment was carried out just as was Exp. 16, except that the p_H was made up to about 7·6, not allowing for the acidity of the tissue, and the muscle was left for about 30 minutes in the *acid* phosphate solution with the pancreatin, before the alkali was added.

	cc. of $N/10$ permanganate required	Succinic acid found in mg. %
Control	(*a*) 244	27·6
Control	(*b*) 260	31·8
With pancreatin	(*c*) 96 (62 % inhib.)	13·8 55 % inhib.
With pancreatin	(*d*) —	13·3 „ „
With digested pancreatin	(*e*) 225 No inhib.	27·1 No inhib.

Exp. 20. The effect of added insulin was tried, 5 mg. freshly dissolved in a little Ringer solution being added to each of samples (*d*) and (*e*). The experiment was carried out just as was Exp. 16 except that the p_H was made up to 8·0, not allowing for the acidity of the tissue.

	cc. of $N/10$ permanganate required %	Succinic acid found in mg. %
Initial	(*a*) 84	5·1
No insulin	(*b*) 220	16·3
No insulin	(*c*) 200	17·8
Insulin	(*d*) 210	16·0
Insulin	(*e*) 226	15·1

Exp. 21. The last experiment was repeated, with the differences that care was taken to prevent the p_H from rising above 7·0, for fear of destruction of the insulin by the alkaline reaction during the experiment, and that nitrogen was used.

	cc. of $N/10$ permanganate required %	Succinic acid found in mg. %
Initial	110	8·8
No insulin	280	27·7
Insulin	304	26·4
Insulin	300	24·3

TIME-RELATIONS OF SUCCINIC ACID PRODUCTION.

Exp. 22. The muscle was removed from the leg of a dog immediately upon the death of the animal, and dropped into a pan sunk in ice. The muscle was minced when it had cooled down to about 8°. Four lots of 50 g. each were weighed out into four flasks, each containing 100 cc. of Na_2HPO_4 solution at p_H 8·4 and KCN. The flasks were placed in an incubator at 33°. An initial example was extracted at once.

	Succinic acid in mg. %
Initial	8·0
1 hr.	17·4
2 hrs.	22·0
3·5 hrs.	26·9
3·5 hrs.	29·4

These results are plotted on the accompanying curve (Fig. 1). The contents of the flasks required some time to attain the temperature of the incubator, so that the earlier part of the curve is somewhat flattened; but, of course, all the samples were under the same conditions.

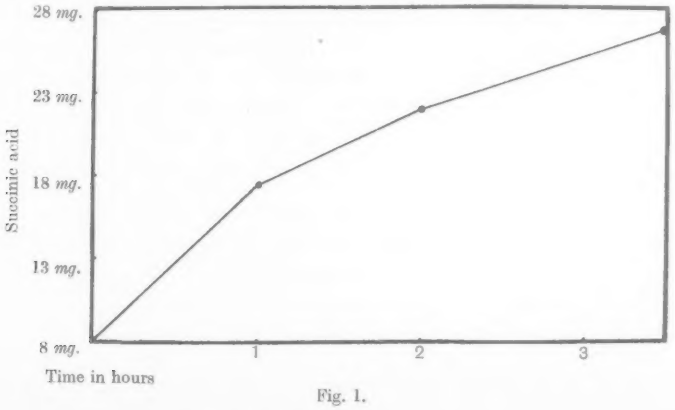

Fig. 1.

Exp. 23. With beef the experiment was carried out at 25°. The muscle samples were weighed out into cooled beakers, and then, when all were ready, transferred as quickly as possible to the flasks of phosphate solution containing 1 : 4000 KCN, already in the bath. In this way the constant temperature was very quickly attained.

	cc. of $N/10$ permanganate required %	Succinic acid found in mg. %
Initial	114	10·0
0·5 hrs.	170	20·3
3·3 hrs.	198	21·4
6·0 hrs.	210	21·3

Exp. 24. The last experiment was repeated.

Initial	120	8·6
20 mins.	160	20·6
40 mins.	192	20·6
2 hrs.	260	22·5

These results show that, in beef, the succinic acid maximum is very quickly reached. The fact that this maximum remains unchanged even after six hours makes it practically impossible that bacterial action can be responsible.

Discussion of results.

The results show formation of succinic acid in all the different kinds of muscle tested, with the possible exception of that of the frog. The very small increase in succinic acid concentration in the pale muscle of the rabbit and in the muscle of the frog is interesting; it has been shown that, after 3·5 hours' incubation the red muscles of the rabbit contain about three times as much succinic acid as do the corresponding weights of white muscle. It is possible that this fact should be correlated with the finding of Batelli and Stern, that the red muscle of the hen and the calf are much more active in the oxidation of succinic acid than the pale muscles of these animals; it may be that in pale muscle the whole question of succinic acid metabolism is much less important. Frog muscle certainly contains a succinoxydase, but as the figures given by Thunberg [1909] for the oxidation of succinic acid in the muscle of the frog are obtained from unwashed muscle, while those of Batelli and Stern [1911] for the rabbit, dog, ox, etc. are with washed muscle, the activity of the frog cannot be compared with that of the mammalian muscle. It is hoped that directly comparable figures may be obtained, and also that the succinic acid formation in other red and white muscles may be determined.

The origin of the succinic acid remains obscure. The result with added glutaminic and aspartic acids shows that the tissue possesses the power of deamination of one or both of these amino-acids (whether hydroxyglutaric or succinic acid is formed is, of course, as yet undecided). But the fact that the muscle could form succinic acid from these amino-acids would not necessarily mean that this was the only source of the acid in the muscle. The extraordinary activity of the succinoxydase makes one inclined to believe that

something more important is involved than the oxidative metabolism of these two amino-acids, especially when it is remembered that they have no specific dynamic action [Lusk, 1913; Atkinson and Lusk, 1918].

The similarity, which has been noticed in the effect of pancreatic preparations on the lactic and succinic acid production, might be interpreted as an indication that succinic acid is involved in carbohydrate metabolism. This view would be much strengthened if it were possible to obtain a trypsin-free extract with the property of inhibiting both succinic and lactic acid formation. Work with this object is now in progress. It seems impossible, however, that succinic acid formation can be a reaction necessarily coupled in any way with the production of lactic acid, for it has been shown that lactic acid is still being formed long after the succinic acid has reached its maximum; and the addition of sugar and toluene may affect the production of one acid and not of the other. All that one could suppose would be that the succinic acid formation is a side-reaction or a concurrent reaction, proceeding at a velocity independent of the velocity of the production of lactic acid.

The very low value of the succinic acid maximum might be explained in several ways: (*a*) lack of precursor; (*b*) lack of co-ferment; (*c*) equilibrium point; (*d*) destruction of enzyme, *e.g.* by accumulation of lactic acid. If the precursor be an amino-acid, the low maximum is easily understood, as the amino-acids are brought to the muscle in the blood-stream, and do not accumulate there. The question of the need of a co-ferment has been considered in the paragraph on pancreatic extracts.

In order to test whether the succinic acid maximum obtained is an equilibrium point determined by accumulation of end-product, it would be necessary to remove the succinic acid formed, and see whether more could be produced to take its place. Preliminary experiments, in which the muscle has been placed alternately in atmospheres of nitrogen and oxygen, have been carried out, and the few results obtained so far, seem to show that the maximum can be repeatedly obtained.

Summary.

1. A method has been described for the estimation of succinic acid, but the reservation is made that any hydroxyglutaric acid in the muscle may be included in the figures given for succinic acid.

2. An increased yield of succinic acid is found after anaerobic incubation in phosphate buffer solutions, of p_H 7·0–8·4, in the muscle of the ox, dog, sheep, rabbit, and possibly of the frog.

3. The yield is increased by adding glutaminic and aspartic acids, but not by adding sugar.

4. The yield is markedly decreased by adding certain pancreatic preparations.

5. In the red muscle of the rabbit, the yield is much greater than in the white.

I wish to express my sincere thanks to Prof. F. G. Hopkins, for his most valuable suggestions and kind interest in the work.

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XLVIII. ON THE FERMENTATION OF THE UNSATURATED DICARBOXYLIC ACIDS. PART I. FUMARIC ACID.

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Both ammonium fumarate and ammonium succinate provide excellent sources of nutrition to a number of bacteria—particularly to *B. pyocyaneus* and *B. fluorescens liq.* Table I shows the nutritional properties of these substances for various types of bacteria.

Table I.

Organism	Ammonium succinate	Ammonium fumarate
Sarcina aur.	Practically no growth after 21 days' incubation	No growth after 21 days
B. pyocyaneus	Extensive surface growths. Thick, almost gelatinous growth after 21 days. Considerable pigmentation after this time	Extensive surface growths. Growth extremely vigorous. Not so much pigmentation as with the succinate
B. subtilis	Little or no growth. No film produced	No growth observable. No film produced
B. fluorescens liq.	Thick brown growth of a jelly-like consistency after 21 days	Excellent growth
B. proteus vulg.	Slow but perceptible growth	Fairly good growth
Staphylococcus aur.	Poor growth	Poor growth
B. prodigiosus	Fairly good growth	Fairly good growth
Timothy grass bacillus	No growth observable	No growth observable
B. coli comm.	Very good growth	Very good growth

The ammonium succinate or fumarate was the sole source of carbon and nitrogen in the media provided for these observations. A concentration of about 1 % was usually employed. It was used in conjunction with a Ringer solution of the following composition: NaCl ·5 g.; K_2HPO_4 ·2 g.; $MgSO_4$ 0·2 g.; $CaCl_2$ 0·1 g.; water, 1 litre; the medium being generally at an initial p_H of 7·4. Twenty-four hour nutrient agar cultures of the organisms were used for inoculation into the medium. The extraordinarily good growths of *B. pyocyaneus* made this organism very suitable for an investigation into the products formed by its fermentative action and for determining its relative rates of utilisation of the succinate and the fumarate.

The products of fermentation of succinic and fumaric acids by B. pyocyaneus.

Aubel [1921] subjected ammonium succinate to fermentation by *B. pyocyaneus* and found that propionic acid was produced. It will be found, however, that other lower fatty acids are also formed, the actual yield of propionic

acid varying considerably with the conditions under which the fermentation is allowed to proceed. If to the ammonium succinate caseinogen be added, a much larger amount of propionic acid is formed than in its absence—the difference not being due to the fermentation of caseinogen alone, as a control experiment showed. In no case is the yield of propionic acid high, and if the fermentation be allowed to proceed too far (for instance to a jelly-like consistency) the actual amount is very small. From the products of fermentation of 30 g. succinic acid which had been neutralised with ammonia and dissolved in 3 litres of water (to which mineral salts in the concentrations given above had been added), about 2 g. of a fairly pure specimen of propionic acid were obtained. The acid was identified by an analysis of the barium salt recrystallised from water (Ba, found 45·43 %; Ba, calculated for hydrated propionate 45·58 %). This was obtained after a fortnight's growth at 37° with an initial p_H of 7·4. Analysis showed that after this time 85 % of the succinate had been utilised. In a number of other experiments on the fermentation of succinic acid the analysis of the volatile acids produced showed a variable barium figure demonstrating the existence of a varying mixture of fatty acids (chiefly lower ones). Small quantities of formic acid were usually found. Fumaric, malic, and other non-volatile acids were especially sought among the products of fermentation, but none was found.

Fumaric acid gives rise to similar products after a fermentation lasting over a fortnight, and it is itself entirely utilised by the end of that time. There seems to be generally a greater proportion of acetic acid among the fatty acids produced than in the case of succinic acid. The crude acids obtained by distillation *in vacuo* were neutralised with baryta and an analysis of the dried barium salts gave Ba 50 to 53 %. Traces of formic acid but no non-volatile dibasic acids were found among the products of fermentation.

In the cases of both succinic and fumaric acids, the fermentations are accompanied by marked increases in p_H, and ammonium carbonate is found in solution. The pigmentation in both cases is not so great as that produced by the growth of *B. pyocyaneus* in a caseinogen digest, and generally appears to be more marked in a succinate medium than in a fumarate. The amount of pigmentation, however, is affected not only by the type of medium utilised and the length of the fermentation, but by such conditions as the nature of oxygen supply, stirring of the medium, and especially by p_H.

The rates of utilisation of ammonium succinate and ammonium fumarate by B. pyocyaneus.

The growth of *B. pyocyaneus* in fumarate and succinate media appears to occur chiefly at the surface of the solution. After shaking, the scum falls below the surface and renders the solution turbid. It is very difficult to obtain a homogeneous emulsion of the organism in such a solution. Hence in determining the rates of utilisation of the media it is inaccurate to use a method

involving the analysis of aliquot parts of the medium at various intervals. Moreover, the medium evaporates to a considerable extent in the incubator, the amount of evaporation depending on the extent of the surface of the solution, so that it is difficult to apply corrections. The following method, which was found to give the most concordant results, was eventually used.

Ten cc. of approximately 2 % ammonium succinate-Ringer solution which had been brought to a p_H of 7·4 were placed in each of 20 test-tubes. These were plugged and sterilised by autoclaving. The ammonium succinate had been prepared by neutralising a strong aqueous solution of succinic acid with ammonia, evaporating, and allowing to crystallise. The dried crystals consisted chiefly of the ammonium salt, the remainder being the acid salt. A series of ammonium fumarate-Ringer tubes was prepared in a similar way.

Nutrient agar-agar slopes were sown with *B. pyocyaneus* and after 24 hours the organisms were emulsified in 0·85 % sterile saline solution, washed by centrifugalisation, and finally made up with the saline solution to a volume which showed just a slight opalescence. This appeared to be fairly homogeneous, and after several hours no deposit of the organism was formed. Into each of the tubes of media 1 cc. of the *B. pyocyaneus* suspension was run by means of sterile pipettes, and the tubes were then incubated at 37°. Each tube was well shaken every morning and at definite intervals the contents of a number of the tubes were analysed. The analyses were done in duplicate.

Estimations of succinate and fumarate in fermentation media.

A method had to be devised which would allow of a fairly rapid and accurate determination of succinic acid in the presence of products of fermentation, protein matter, phosphates, chlorides, etc. The following method proved to be the most satisfactory of a number of attempts. The medium containing the succinate is washed into a conical flask and about 1 g. lime added. The mixture is boiled gently for two minutes. It is then cooled, filtered, and the residue washed three or four times with small quantities of water. The total filtrate and washings should not occupy a volume of more than 40 cc. This solution is quite clear and apparently free from protein matter and from phosphates. A few drops of phenolphthalein indicator are added, the solution titrated with approximately $N/5$ sulphuric acid until just acid, and titrated back with a drop or two of dilute alkali until the solution is faintly pink. Silver nitrate in concentrated solution and in slight excess of that necessary to precipitate the whole of the chlorides and succinate is added, the solution allowed to stand a few minutes, filtered and the precipitate washed until free from silver. The precipitation should be carried out in a dark part of the laboratory and the washing should be done as quickly as possible. The precipitate is washed into a white porcelain basin, a little nitric acid added to dissolve the succinate, then a few drops of ferric alum indicator added and the solution finally titrated with standard potassium thiocyanate solution in the usual manner. An error of 2 % was the largest experienced in numerous

analyses of succinate solutions of known composition where the succinate varied in concentration between 5 % and 0·02 %. At a concentration of 0·01 % or less the method is less reliable, but an accuracy of 5 % can usually be obtained. Care must always be taken in precipitating and washing the silver succinate.

This method of silver precipitation may also be used with accuracy for the estimation of fumaric acid. The products of fermentation of succinic and fumaric acids by *B. pyocyaneus* are not precipitated by silver under the conditions occurring in this method of analysis.

The results of a parallel series of fermentations of succinic and fumaric acids are expressed graphically in Fig. 1.

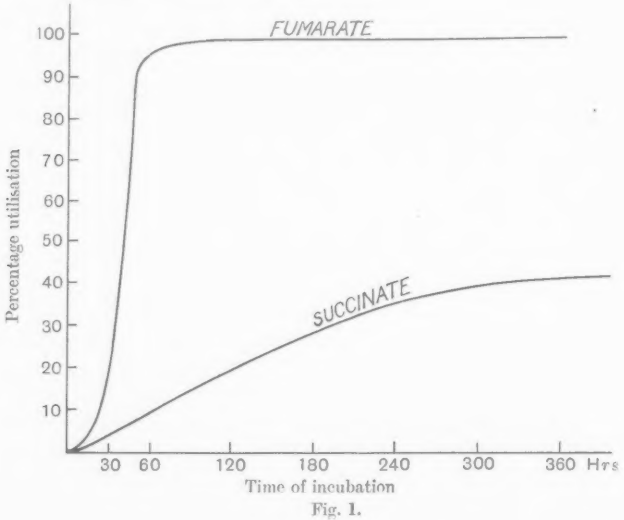


Fig. 1.

This is typical of a large number of parallel series of fermentations. The rate of fermentation of ammonium succinate varies considerably with the number of organisms inoculated and the initial p_H, but generally not more than 50 % of the succinate is utilised within the first week of fermentation. Under these conditions, however, a fumarate medium of the same percentage composition as the succinate is almost entirely utilised within two or three days. In both cases there is a rise in p_H; in the fumarate the p_H may rise to 11 or over, but in the succinate the p_H has not been observed to rise above 10. Ammonium carbonate is produced in both cases. The rates of fermentation, especially in the case of the succinate, are accelerated by bubbling air or oxygen through the medium. Under this condition an ammonium succinate medium may be utilised to the extent of 70 % within a week and a fumarate medium utilised to the extent of 95 % within 36 hours.

In spite of the wide differences in the rates of utilisation of ammonium succinate and ammonium fumarate by *B. pyocyaneus*, the actual amounts of growth of the organism in both are about the same. This was shown by comparing the volumes of centrifugalised deposits of organism. An attempt was made to count the number of organisms by the usual procedure of plating-out, but owing to the coherent nature of the bacillus this was abandoned. The pigmentation of a fumarate medium which had just been completely utilised (*i.e.* within two or three days of inoculation) was slight, but it increased considerably later. On the whole a parallel succinate fermentation showed greater pigmentation.

Properties of a fumarate medium after fermentation for 48 *hours.*

The next step in the work was to ascertain the nature of the constituents of the medium after the fumaric acid has just been utilised, *i.e.* after roughly 48 hours' fermentation. It was obvious that the fumaric acid had been replaced by a reactive material, for the solution was capable of reducing silver oxide to silver, mercuric chloride to mercurous chloride, and picric acid to picramic acid. It could reduce molybdates and tungstates to the blue lower oxides, but did not react with Fehling's solution. Colour tests capable of detecting small quantities of this reactive material were sought and two were found which were of considerable use in the early part of the work. A third was subsequently discovered which facilitated the isolation of the material. The two tests referred to were:

(1) *Tryptophan test.* If 10 cc. of a 1 % solution of ammonium fumarate in a Ringer solution containing chalk in suspension (the p_H being approximately 8·0) be inoculated with a loopful of *B. pyocyaneus* and allowed to ferment for 24 hours, the solution gives a characteristic colour reaction with tryptophan. To 1 cc. of this solution in a test-tube, a little tryptophan is added and conc. H_2SO_4 slowly poured down the side of the tube; a bluish violet coloration is produced and this slowly spreads upwards—not into the acid layer as with the glyoxylic test. If fermentation be allowed to proceed for a week or more, the solution gives a deep brown ring instead of a bluish violet. If a solution capable of giving the violet ring be boiled vigorously, it will no longer (on cooling) give this ring. If chalk be omitted from the medium, the initial p_H still being approximately 8·0, the violet ring is not always given but is replaced by a brown one. If, as was subsequently done, the fermenting medium be continually aerated or bubbled with oxygen, a violet ring is rarely given but usually a deep brown one. This test is not given by a succinate medium which has been fermented by *B. pyocyaneus*, nor is it given by a fumarate medium which has been fermented by *B. coli*.

(2) *Nitroprusside test.* If in 1 cc. of a 1 % ammonium fumarate-Ringer solution which has been fermented for 36 hours with *B. pyocyaneus*, a crystal of sodium nitroprusside be dissolved, and to the solution solid ammonium sulphate and finally strong ammonia be added, a blue or bluish-green colora-

tion gradually appears in the vicinity of the ammonium sulphate crystals. The colour becomes most intense after standing at room temperature for a quarter to half an hour. The best tests are obtained when the fumarate medium has been inoculated with a loopful of fresh culture of *B. pyocyaneus* and has been continually aerated. A medium which has been boiled will give the test provided that the medium was originally capable of giving a strong reaction. If, however, the fermentation has been carried out with an insufficient oxygen supply, the reaction is usually a weak one and after boiling the test is hardly given. The colour of this test seems to be identical with that given by pyruvic acid. It disappears on standing, as is usual with nitroprusside tests. It is readily given when the blue-violet ring of the tryptophan test has been replaced by a brown one.

The only organisms found so far which yield the tryptophan and the nitroprusside tests after fermentation on a fumarate medium are *B. pyocyaneus* and *B. fluorescens liq.* No organisms have yet been found to give these tests after fermentation on a succinate medium. The tests are not given by the pigment of *B. pyocyaneus* or by the organisms alone.

With these tests for the reactive constituent of the medium, attempts were made to extract or isolate it. It was found in the first place to be non-volatile at or below the boiling-point of water. Distillation at 100° or at low temperatures *in vacuo* failed to carry over the reactive material. Moreover, prolonged boiling destroyed it. If the medium were evaporated to dryness *in vacuo*, the residue only gave a faint nitroprusside reaction—not nearly as great as that expected had the evaporation brought about a concentration of the material without loss due to decomposition. The addition of phosphoric acid to a reactive medium did not destroy its activity but distillation in steam of the acid solution failed to carry over the material. Ether extraction of a slightly acid reactive medium carried out continuously over 36 hours failed to extract more than a trace of material giving a nitroprusside reaction. Moreover, the aqueous portion after thorough extraction with ether still yielded a fairly good test. On adding a solution of phenylhydrazine hydrochloride to a few cc. of the reactive medium saturated with sodium acetate, no precipitate of a hydrazone appeared. All attempts to precipitate the reactive material with metals failed. Other attempts at isolation by different solvents, by distillation, etc. were focussed on the residue left after evaporation *in vacuo*, and it was obvious after many failures to isolate either the reactive material or a derivative of it that a different mode of attack was necessary.

These attempts at isolation were all carried out on 1 % ammonium fumarate dissolved in Ringer solution (at a p_H varying between 7·4 and 8·0) which after inoculation had been incubated at 37° for two or three days. It was evident that this method of inoculating 500 to 1000 cc. of the medium and incubating it yielded only very small quantities of the reactive constituent. It was evident, too, that the constituent was probably a ketonic acid and that its isolation by ether extraction or as a hydrazone was being hindered not

only by its low concentration but probably by the presence of other substances in the medium.

Two courses were possible, (1) to determine the conditions which would bring about an increase in the concentration of the reactive constituent; (2) to guess the nature of the constituent from the data already available, to determine experimentally the precise conditions for its isolation from a medium made up roughly to correspond with a fermented fumarate medium, and then to apply these conditions to its isolation from the original medium.

The work at this point was considerably facilitated by the discovery of a characteristic reaction given by the above medium in the presence of guaiacol and sulphuric acid.

Guaiacol test. If to 1 cc. of a 1 % ammonium fumarate-Ringer solution which has been fermented by *B. pyocyaneus* for 36 hours, a few drops of an alcoholic solution of guaiacol be added and conc. H_2SO_4 poured cautiously down the side of the test-tube, a characteristic band of colours is formed at the surface of separation. There is a thin crimson band nearest the water layer. Underneath this is a wider band which is deep carmine and fades into pink as it penetrates the sulphuric acid layer. This test is very sensitive—more so than the nitroprusside test—and there is no indecision as to its being positive or not as there may be with a weak nitroprusside test. The intensities of the guaiacol and nitroprusside tests proceed parallel to one another as fermentation progresses. Both are most intense at the same period of fermentation and both disappear after a few days' fermentation.

The guaiacol test is very characteristic of this fermentation. It is not produced in a succinate medium fermented by *B. pyocyaneus* and the only organisms among those hitherto examined which produce it in a fumarate medium are *B. pyocyaneus* and *B. fluorescens liq.* The only substance found so far which gives a guaiacol reaction identical with that of a fermenting fumarate is pyruvic acid, but a wide range of substances has not yet been tested. Acetone gives a yellow ring. Acetaldehyde produces a white diffused layer underneath which is a scarlet band followed by a clear yellow one penetrating into the sulphuric acid. Formaldehyde gives a very thick white diffused layer over a deep claret band which penetrates into the acid. All these tests are given at low concentrations and the distinct differences in colours are best seen at these concentrations (about 1 part in 1000 and less).

These colour reactions, together with the reducing properties, exhibited by the medium after a relatively short period of fermentation, made it extremely probable that pyruvic acid was produced during the fermentation. A number of experiments were then undertaken to determine under what conditions pyruvic acid in dilute solution (approximately 0·2 %) could be isolated as such or as a known derivative from a medium made up roughly to correspond with a fermented medium. Eventually it was found that the most satisfactory method was to precipitate the pyruvic acid as the *p*-nitrophenylhydrazone, but certain precautions must be taken to ensure the precipitation.

It was necessary that bacterial matter should first be removed from the medium, that any carbonate originally in the medium should be removed by the addition of a little syrupy phosphoric acid, and that only a few drops (not excess) of a *freshly made* saturated solution of *p*-nitrophenylhydrazine in strong acetic acid should be used for the precipitation. It was advisable always to add a little syrupy phosphoric acid for this seemed to facilitate the precipitation considerably. There was, however, a limiting concentration of pyruvic acid under which no precipitation could be obtained and since the strength of the guaiacol and nitroprusside tests showed that this concentration was approximately that obtained in a fermenting fumarate medium it was necessary to find conditions to increase this concentration in the medium in order to ensure precipitation.

Isolation of pyruvic acid as the p-*nitrophenylhydrazone.*

It was finally found that under the following conditions a maximum yield of the reactive substance is formed.

1. A 1 % solution of sodium fumarate instead of ammonium fumarate should be fermented. The nitrogen is supplied in the form of ammonium chloride (0·4 %).

2. The initial p_H of the medium should be approximately 7·4 and no chalk should be added.

3. *The medium must be continually aerated.*

4. It must be initially inoculated with a loopful of a 24 hour agar-agar culture of *B. pyocyaneus.*

5. The fermentation should not be carried on for more than two days. Generally 30 hours' fermentation or even less gives the most intense reactions. When a film of the organism no longer forms at the surface of the medium, the change of the fumaric acid is generally completed.

6. Large quantities of the medium should not be fermented at a single time. 100 cc. and 50 cc. lots are most satisfactory and these are fermented in conical flasks so that as large a surface as possible is obtained.

A medium treated in this way gives most intense nitroprusside and guaiacol reactions.

To 10 cc. of this medium is added about 1 g. chalk. The solution is thoroughly mixed and filtered. The filtrate is practically clear and free from protein matter. One cc. of syrupy phosphoric acid is added to the filtrate and this is slightly warmed until CO_2 is no longer evolved. The solution is cooled and a *freshly prepared* saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid is added a drop at a time, with shaking. Not more than 0·5 cc. is added. On standing a yellow precipitate appears. This is filtered on a Buchner funnel, washed with a little glacial acetic acid and subsequently with water. The precipitate is recrystallised from hot dilute alcohol, from which it separates in

sheaves of yellow needles. Its melting-point is 219° which is identical with that of the *p*-nitrophenylhydrazone of pyruvic acid [Hyde, 1899] and its melting-point is not changed by the admixture of a pure specimen of this hydrazone.

There is little doubt therefore that the substance responsible for the reactive properties of a fumarate medium which has been inoculated with *B. pyocyaneus* is pyruvic acid.

Pyruvic acid has itself been subjected to putrefactive fermentation and acetic acid has been the main product formed [Neuberg, 1914, 2]. The formation of acetic acid by lengthy fermentation of fumaric acid probably proceeds therefore through pyruvic acid.

The yield of pyruvic acid is smaller in an ammonium fumarate medium than in one containing a relatively small quantity of ammonia. There is evidence that in the former medium complexes are formed, which are probably condensation compounds of pyruvic acid with ammonia. The p_H of the medium becomes very high; in this state of alkalinity pyruvic acid readily produces condensation products with ammonia.

No substances other than pyruvic acid, the lower fatty acids, bacterial matter, and CO_2 were found in measurable quantity. Hydrogen has not yet been sought. The following were sought and not found: acetaldehyde and formaldehyde; lactic, acrylic, glycollic, malic, tartaric and malonic acids; ethyl alcohol and methylacetylcarbinol. Methods for acetaldehyde fixation in the fermenting medium have not yet been tried.

The effects of various organisms on an ammonium fumarate medium.

One loopful of a fresh culture of each of a number of organisms was inoculated into 1 cc. of a 1 % ammonium fumarate-Ringer solution at p_H 7·6, and incubated at 37°. They were examined by the guaiacol and nitroprusside tests after (1) 40 hours, and (2) six days from the time of inoculation—the fermentation tubes being well shaken each morning. The results are summarised in Table II.

Table II.

	(1) After 40 hours			(2) After 6 days		
Organism	Growth	Nitroprusside test	Guaiacol test	Growth	Nitroprusside test	Guaiacol test
B. pyocyaneus	Very good	+ + +	+ + +	Very good	Very feeble, positive	Very feeble
B. fluorescens liq.	,,	+ + +	+ + +	,,	–	–
B. coli communis	Good	–	–	,,	–	Slight test indicative of aldehyde (not of pyruvic acid)
B. subtilis	? Growth	–	–	? Growth	–	–
B. prodigiosus	Fair growth	–	–	Fairly good	–	Slight test indicative of aldehyde (not of pyruvic acid)
B. proteus vulg.	Slight growth	–	–	Slight	–	,,
Staphylococcus aur.	? Growth	–	–	,,	–	,,

The powerful action of *B. pyocyaneus* and *B. fluorescens liq.* compared with the other bacteria is most marked and the guaiacol test provides an excellent method for the differentiation of these organisms.

No growth of *B. pyocyaneus* (which is a facultative anaerobe) could be obtained anaerobically in an ammonium fumarate or succinate medium.

Oxygen uptake and carbon dioxide output.

Investigations were made to determine the amount of oxygen taken up and the quantity of carbon dioxide produced during the fermentation of fumaric acid by *B. pyocyaneus*.

For this purpose use was made of an apparatus designed by Stephenson and Whetham [1923]. This consisted of an incubation flask with an air condenser tube. Air, after passing through wash-bottles containing potash and conc. H_2SO_4, was aspirated slowly through the incubation flask. It passed out through the air condenser tube and then through two weighed pumice-conc. H_2SO_4 absorption tubes and a weighed potash bulb. The apparatus was disconnected at intervals and each part cooled and weighed. The gain in weight of the whole apparatus gave the total oxygen uptake. The gain in weight of the potash bulb plus that of the sulphuric acid absorption tube attached to it gave the total carbon dioxide output.

The following figures give the result of a typical blank experiment:

	Initially	After 24 hours' aeration
Wt. of incubation flask	149·7765 g.	149·7550 g.
Wt. of first H_2SO_4 absorption tube	45·3690	45·3905
Wt. of second H_2SO_4 absorption tube	43·4934	43·4945
Wt. of potash bulb	62·1096	62·1120
	300·7485	300·7520

There is generally an error of not more than 5 mg., and this may be regarded as small compared with the total oxygen uptake and carbon dioxide output in the course of a fermentation.

The following gives the result of a fermentation carried out on 50 cc. of a 1 % fumaric acid as ammonium fumarate medium, p_H 7·4.

	After 20½ hours' fermentation	After 42½ hours' fermentation	After 64½ hours' fermentation	After addition of phosphoric acid and aeration
Carbon dioxide output	·0384	·1224	·1529	·2129
Oxygen uptake	·0684	·0754	·0744	·0744

The total oxygen uptake is 0·0744 g. and the total CO_2 output is 0·2129 g.

The oxygen is absorbed to the greatest extent during the first 30 hours of fermentation. Even after 20 hours, over 90 % of the total oxygen absorbed has been taken up. After two days oxygen is taken up very slowly and in certain cases, as in the one quoted, appears to cease to be absorbed. This is partly due to the retardation of growth brought about by the increased p_H of the medium, and partly due to the fact that all the fumaric acid has been utilised. Carbon dioxide appears to be evolved at a rate similar to that of

oxygen uptake. The actual values of carbon dioxide output must be taken in conjunction with the increase in p_H. At the end of the experiment a little phosphoric acid is added to the incubation flask, which is thoroughly aerated, and the remaining carbon dioxide is thus driven over into the potash bulb which is re-weighed. The total carbon dioxide produced is estimated at the end of the experiment. The ratio $\frac{\text{carbon dioxide output}}{\text{oxygen uptake}}$ is in the example quoted above 2·86. A second example is given below (50 cc. of a 1 % fumaric acid medium were used).

	After 22½ hours' fermentation	After 47 hours' fermentation	After 98½ hours' fermentation	After addition of phosphoric acid and aeration
Carbon dioxide output	·0305	·1760	.1965	·2370
Oxygen uptake	·0822	·0915	·0870	·0870

Total oxygen uptake = 0·0870 g.

Total CO_2 output = 0·2370

$$\text{Ratio } \frac{CO_2 \text{ output}}{O_2 \text{ uptake}} = 2{\cdot}72.$$

A third experiment gave the ratio $\frac{CO_2 \text{ output}}{O_2 \text{ uptake}} = 2{\cdot}61$, and we have as the average of these three results $\frac{CO_2 \text{ output}}{O_2 \text{ uptake}} = 2{\cdot}73$. If we assume the early part of the fermentation of fumaric acid to follow the equation

$$\begin{matrix} CH.COOH \\ \| \\ CH.COOH \end{matrix} + O = \begin{matrix} CH_3 \\ | \\ CO.COOH \end{matrix} + CO_2,$$

we have the ratio

$$\frac{CO_2 \text{ output}}{O_2 \text{ uptake}} = \frac{44}{16} = 2{\cdot}75.$$

Moreover, since 116 g. of fumaric acid will require an uptake of 16 g. of oxygen for this reaction, the amount subjected to fermentation, *i.e.* 0·5 g. will require 0·069 g. of oxygen. Experiment shows that a little more than this amount of oxygen is utilised, and this is probably used in the oxidation of pyruvic acid to acetic acid:

$$\begin{matrix} CH_3 \\ | \\ CO.COOH \end{matrix} + O = \begin{matrix} CH_3 \\ | \\ COOH \end{matrix} + CO_2,$$

where the ratio CO_2/O_2 is the same as in the previous reaction. If this be the case, then the difference in oxygen uptake between the experimental and the theoretical should be equivalent to the amount of acetic acid produced. This may be shown to be approximately the case. In the second example quoted above, the amount of fatty acids present calculated as acetic acid was 0·077 g. The theoretical quantity was 0·071 g.

The Balance Sheet.

An effort was made to construct a balance sheet between the fumaric acid and the products of its fermentation. This involved determining the quantity of pyruvic acid present. A number of methods were tried. The most

satisfactory—in that it gave consistent results—depended on the oxidation of the pyruvic acid with silver oxide and the estimation of the acetic acid produced.

50 cc. of the medium containing the pyruvic acid were treated with 1 g. of lime, warmed and filtered. 2 g. of freshly prepared, well washed, silver oxide were added to the filtrate and the solution was boiled gently for a quarter of an hour or more. A silver mirror usually formed. The solution was filtered, the precipitate washed, and the total amount of acetic acid in the filtrate and washings determined. This was carried out by acidifying the solution with glacial phosphoric acid and distilling *in vacuo*, the distillate being passed into a known quantity of standard baryta solution. The baryta was titrated after the distillation and the amount of fatty acid distilled over thus determined. Another 50 cc. of the same medium were acidified and distilled without previous treatment with silver oxide. This gave an estimation of the original quantity of fatty acids present; the difference between this figure and the previous one gave the quantity of acetic acid derived from the pyruvic acid and a simple calculation served to determine the amount of pyruvic acid originally present.

The amount of bacterial substance was estimated roughly by filtering through asbestos on a Gooch crucible (which acted as a fairly efficient filter) and drying the bacteria in a steam oven to constant weight. They were then placed (with the asbestos) in a combustion tube, burned in a current of oxygen, and the amount of carbon dioxide produced was estimated.

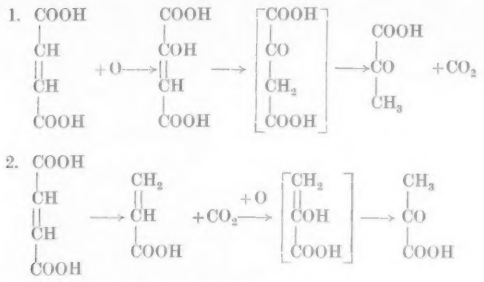
The following is an analysis of the products of 100 cc. of a 1 % fumaric acid medium (the fumaric acid being present as the sodium salt and ammonia as 0·4 % ammonium chloride). The fermentation had been allowed to proceed for 20 hours. 92 % of the fumaric acid had been utilised.

		Calculated in terms of CO_2	Fumaric acid utilised expressed in terms of CO_2
Acetic acid (being total fatty acids expressed as acetic acid) ...	=0·0922 g.	0·1352	
Pyruvic acid	=0·287	0·431	
CO_2 output	=0·550	0·740	
CO_2 from the organism	=0·190		
		1·3062 g.	1·398 g.

Thus at least 90 % of the fumaric acid utilised can be accounted for; the remainder is probably in the form of complex condensation products. Generally the yield of pyruvic acid from 1 g. of fumaric acid under the best conditions is about 0·20 g.

Discussion.

Since pyruvic acid is formed in the fermentation of fumaric acid by *B. pyocyaneus*, our first enquiry turns on the nature of the intermediate compound which is presumably formed. There are two possibilities, viz.:



The following evidence is available:

1. Oxalacetic acid is known to be easily decarboxylated by yeast [Mayer, 1913]. It is fermented by putrefying bacteria to acetic acid [Neuberg, 1914, 2]. Experiments are now in progress on the growth of *B. pyocyaneus* on oxalacetic acid.

2. The theoretical quantity of carbon dioxide required by the decarboxylation of either fumaric or oxalacetic acid is eliminated. The organism must, therefore, use the decarboxylated molecule for growth. Hence it would be expected that if decarboxylation of fumaric acid were the first step, growth would occur on acrylic acid. It has been impossible so far to obtain any growth of *B. pyocyaneus* on a medium in which acrylic acid forms the sole source of carbon; neither has acrylic acid been found among the products of fermentation of fumaric acid. Growth does occur with pyruvic acid as sole source of carbon.

3. No oxalacetic acid has been found, but experiment shows that the rate of decarboxylation (*i.e.* of CO_2 output) is practically equal to the rate of oxygen uptake, so that the oxalacetic acid is decomposed about as quickly as it is formed.

4. Oxalacetic acid has been previously postulated as the first product formed in the utilisation of fumaric acid by *Aspergillus niger* [Clark and Raistrick, 1919].

On the whole the evidence is in favour of oxalacetic acid (or its enol isomer) being the first product formed in the utilisation of fumaric acid. This phenomenon adds another example to the list of biological oxidations which we find, as yet, impossible to repeat *in vitro*. Neuberg [1914, 1] has been able to obtain acetaldehyde from fumaric acid by oxidation with hydrogen peroxide in the presence of iron. It is possible that oxalacetic acid is actually produced here as an intermediate compound, and that this then breaks down to acetaldehyde and carbon dioxide.

The actual mechanism of decarboxylation of oxalacetic acid (assuming that this is the process which occurs) is of interest. Two views are possible—that decarboxylation occurs at the carboxyl group next the ketonic group, or at the carboxyl group next the $> CH_2$ group. On this point there is at present little evidence. Experiments on substituted fumaric acids are about to be carried out and these should give data for a definite conclusion.

Our next enquiry turns on the differences exhibited by various bacteria in a fumarate-Ringer medium. *B. pyocyaneus* and *B. fluorescens liq.*, as possibly might have been expected, are closely related in their behaviour, but *B. coli*, *B. prodigiosus* and *B. proteus* show apparently considerable differences from the highly pigmented bacteria. It would be desirable to know whether the difference is due to the absence of specific enzymes which are present in *B. pyocyaneus* and *B. fluorescens liq.*, or whether it is due to differences in the relative rates of the different reactions constituting the whole process—the process being the same with all these organisms.

It will not be out of place to mention at this point the results of certain experiments which bear directly on the fermentative properties of *B. pyocyaneus*. These experiments, however, are purely preliminary, and will be discussed in detail in a subsequent communication.

If *B. pyocyaneus* be grown in considerable quantity on an ammonium fumarate medium, centrifuged, well washed with normal saline, and finally ground up thoroughly with acetone and re-washed to remove acetone, these bacteria show no power to oxidise fumaric acid to pyruvic acid. When half a gram of bacteria treated in this way were placed in a fumarate-Ringer medium and thoroughly aerated, no trace of pyruvic acid was found for at least 12 hours. A good guaiacol test then resulted but it was found that the medium after this time contained living *B. pyocyaneus*. The pyruvic acid was thus probably derived from the growth of a few bacteria which had not succumbed to the treatment with acetone.

Experiments carried out by Miss Stephenson, Miss Whetham and the author show that *B. pyocyaneus* well washed with normal saline, and dried at $-6°$ with absolute alcohol and ether, has no power of oxidising fumaric acid to oxalacetic or pyruvic acids. The dried organism, it is interesting to note, resembles muscle tissue which has been similarly treated in that it reduces oxidised glutathione with considerable rapidity. It yields, itself, a slight nitroprusside sulphydryl reaction which is not increased by warming the organism (thus differing slightly from muscle tissue). A large quantity of *fresh B. pyocyaneus* grown on a caseinogen digest, centrifuged, and well washed with normal saline, shows no signs of oxidising fumaric acid within a few hours—though the quantity of organism placed in the fumarate medium is far in excess of the amount formed from a small inoculation of the organism after complete utilisation of a similar amount of fumarate medium. Fresh *B. pyocyaneus* seems to show a distinct resemblance to fresh muscle tissue in its action on succinic acid. The rate of reduction of methylene blue by the fresh organism is considerably accelerated by the presence of sodium succinate. Sodium fumarate has no effect. (If the fresh organism be boiled, its power of reducing methylene blue is destroyed.) From analogy with muscle, we should expect the succinic acid to be oxidised to fumaric acid, the methylene blue acting as hydrogen acceptor. In other words, when succinic acid is fermented by *B. pyocyaneus*, we should expect the main course of fermentation to proceed

through fumaric acid and pyruvic acid to acetic acid—the two intermediate acids being relatively quickly dealt with by the organism. This would necessitate the uptake of a considerable quantity of oxygen (three atoms per molecule of succinic acid). One experiment shows that actually about 90 % of the quantity theoretically required is taken up.

B. coli resembles *B. pyocyaneus* in its ability to reduce oxidised glutathione to the sulphydryl form and to accelerate the reduction of methylene blue in the presence of sodium succinate but not in presence of sodium fumarate.

The apparent refusal of fresh washed *B. pyocyaneus*, or the "resting" organism, quickly to oxidise fumaric acid to pyruvic acid is unexpected. It seems possible that the actual process of growth—of protein synthesis—induces chemical changes in the nutrient medium whose velocities may differ considerably from those of the changes induced by the "resting" organism. The "resting" organism and the washed and dried organism (in the case of *B. pyocyaneus* and *B. coli*) have similar properties to fresh and to washed and dried muscle tissue respectively. It is interesting to note that in the quantitative work on the fermentation of fumaric acid an almost theoretical quantity of carbon dioxide is eliminated. It would seem that the organism does not take up the carbon dioxide produced in decarboxylation, but uses the remaining part of the decarboxylated molecule for its synthetic operations. The actual yield of pyruvic acid is much lower than that expected from a theoretical decarboxylation. The problem of the actual mechanism by which the oxidation of fumaric acid is accomplished, and of its *apparent* connection with an actively working synthetic machinery in the organism, is one whose elucidation should throw considerable light on the metabolism of *B. pyocyaneus*.

Summary.

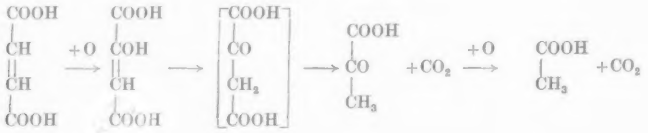
1. Both succinic and fumaric acids are fermented by *B. pyocyaneus* to give mixtures of the lower fatty acids—in the case of fumaric acid chiefly acetic acid.

2. The rate of utilisation of fumaric acid by *B. pyocyaneus* is much more rapid than that of succinic acid, a 1 % solution of ammonium fumarate being entirely utilised in 30 hours. The rate of utilisation in both cases is accelerated by aeration. Ammonium carbonate is produced with increase of p_H. Details of the methods of analysis of the succinate and fumarate media are given.

3. Pyruvic acid can be isolated in the form of the *p*-nitrophenylhydrazone from a fermenting fumarate medium and is responsible for two colour tests—with nitroprusside and with guaiacol—which are given by this medium. These two tests provide an excellent method of differentiating *B. pyocyaneus* and *B. fluorescens liq.* (both of which give the tests) from other bacteria which are capable of growing on a fumarate medium.

4. Investigations have been made on the quantity of oxygen taken up and carbon dioxide produced in the fermentation of a known amount of

fumaric acid. It has been shown that these are practically the theoretical quantities deduced on the basis that the following scheme represents the main course of the fermentation of fumaric acid.



5. A balance sheet between fumaric acid and the products of its fermentation is given, in which over 90 % of the former can be accounted for.

6. The results of a number of preliminary experiments dealing with the relative differences in fermentative properties of the (*a*) washed and dried organism; (*b*) washed fresh, or "resting" organism; (*c*) growing organism, are discussed.

The author wishes to express his indebtedness to Prof. F. G. Hopkins, F.R.S., for the latter's interest, encouragement and help in this work, and to the Scientific and Industrial Research Department for a grant which was held during the early part of this work.

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XLIX. A SIMPLE ELECTRIC THERMOREGULATOR.

By SAMUEL CLEMENT BRADFORD.

From the Science Museum, S.W. 7.

(*Received February 16th, 1924.*)

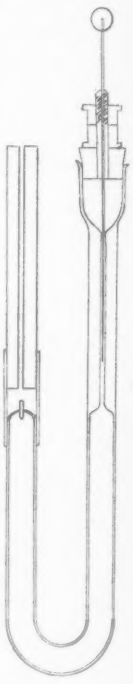
A CONVENIENT, self-contained electric thermostat that will run with a minimum of attention for periods of three months or more has been described previously [Bradford, 1922, 1923]. The apparatus is now obtainable from Messrs Gallenkamp for the moderate sum of ten guineas, and needs merely to be connected to the continuous, or alternate current, supply to be ready for use. The high resistance magnetic cut-out for the heating circuit has unfoulable carbon contacts, and works with a feeble current tapped from the main circuit. During three years' work with several instruments of this type, a variety of thermoregulators has been tried. The ordinary spiral type of toluene regulator is cumbersome and not easy to change from one temperature to another. Perhaps the simplest regulator on the market is a metal one with platinum contacts obtainable from Messrs Gallenkamp. If a drop of heavy oil be placed on the contacts, and the depth of immersion kept constant, this regulator is capable of maintaining the temperature within one-hundredth of a degree for periods extending to six hours. Unfortunately, the apparatus proved subject to variations, due to changes in the humidity of the air or intensity of the current, causing temperature fluctuations of alarming magnitude.

It was, however, interesting to note that the great accuracy during short periods, of which this regulator was capable, was attained by movements of the contacts amounting to only 0·2 mm. per degree. This suggested that the comparatively enormous expansion, provided in thermoregulators with toluene or calcium chloride solution, is altogether unnecessary for electric apparatus, though it may be needed for gas regulation.

When the expansion was reduced to about 0·5 mm. per degree, by dispensing with the toluene, the total movement of the mercury thread in expanding from room temperature to the wished-for temperature was only a few centimetres, and it became possible to use a graduated wire-contact, which could be set instantly at any point desired.

The figure shows a suitable form of such a thermoregulator, two-thirds natural size. Mercury only is contained in the wider portion of the U-tube, which is made of thin-walled glass tubing about 0·6 cm. diameter internally. The closed end

of this tube carries a short piece of platinum wire to form one terminal. One connecting wire from the high resistance magnets of the relay is inserted through the separate capillary tube shown above and makes contact with the platinum wire through a drop of mercury contained in the short piece of rubber tube that serves to connect the capillary tube to the closed U-tube. The capillary tube must not be allowed to press against the end of the U-tube, or there is danger of cracking it. Instead of a rubber connection, the capillary tube may be made in one piece with the U-tube, and the platinum wire inserted through an internal seal.



The other end of the U-tube is joined to a capillary tube of from 1·0 to 1·1 mm. bore, which terminates in a cup, through which the mercury may be poured until it rises a few centimetres in the capillary tube. The other leading wire from the relay circuit is connected to the brass terminal carried by the rubber cork that fits the cup. The adjustable contact is made from No. 22 s.w.g. nichrome wire to slide friction-tight through the screw of the terminal.

The contact has a needle-point at one end, and a knob of vulcanised fibre at the other. It is graduated in millimetres, from within 2 cm. of the point to 1 cm. of the knob, every fifth division being marked with red paint or otherwise suitably distinguished. The top of the screw of the terminal is bevelled to facilitate reading the divisions of the wire.

The apparatus is calibrated by observing the change of temperature of the thermostat corresponding to a somewhat large movement of the wire. The number of degrees corresponding to a division of the wire may vary from about 1·5 to 3·0, according to the dimensions of the apparatus. With the aid of a lens, the wire may then be adjusted instantly so that the thermostat will settle down within 0·05° of any temperature desired and vary no more than 0·01°.

Since a movement of only 4 cm. corresponds to a temperature change of 60° or more, the mercury need never sink below the capillary tube. The extreme simplicity and ease of adjustment make the instrument particularly suitable for the use of students, or when a series of observations needs to be made at stated temperature intervals. It has the advantage of taking up very little space in the thermostat. It may be laid flat without the mercury running out. If the mercury becomes dirty the apparatus may be cleaned in a few moments by raking out the foul mercury from the capillary tube with a wire and cleansing the tube with cotton moistened with alcohol, followed by dry cotton, and pouring in fresh mercury. One disadvantage only must be mentioned, and this is common to every thermoregulator depending on a mercury contact. The continual sparking at the make and break gradually causes the top of the

mercury in the capillary tube to become foul. The evil is reduced to a minimum if only a small current is used in the relay circuit, as in the thermostat already described. In this case the apparatus will run without fouling for from two to three weeks. If a longer run is necessary, a condenser may be connected across the spark gap, or, if a single temperature variation of about 0·05° is permissible, a fresh thermoregulator may be substituted. These are so easy to make that several may be kept ready for use. Further improvement may be sought in the possibility of increasing still more the sensitiveness of the relay. The thermoregulator can be obtained from Messrs Gallenkamp.

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L. NECESSARY CONDITIONS FOR TESTING WITH THE REAGENT FOR VITAMIN C.

(ANSWER TO H. D. KAY AND S. S. ZILVA.)

By NIKOLAI BEZSSONOFF.

From the Laboratoire de Chimie Biologique de Colombes, France.

(*Received February 20th, 1924.*)

Kay and Zilva [1923], having studied the application of my reagent to the identification of vitamin C, found that the majority of antiscorbutic liquids gave the coloured reaction, but that a preparation obtained from lemon juice did not give it, though this preparation tested on animals still contained from 1/3 to 1/4 of the vitamin C present in the initial lemon juice. Secondly, these authors obtained a positive blue reaction with a commercial preparation obtained from yeast, "marmite," and also a positive reaction with the aqueous extracts of brewer's top fermentation yeast, first washed and dried at 37°. Kay and Zilva note that gentle oxidation, proved to be sufficient to destroy the vitamin C contained in lemon juice, also brings about the disappearance of the blue reaction, but they explain these facts by supposing the presence of a substance differing from vitamin C, which is destroyed by oxidation, just like this vitamin. We must add [Bezssonoff, 1922] that the action of oxidising enzymes and that of prolonged heating in the autoclave at 120° lead to the simultaneous disappearance of the blue coloration and of the antiscorbutic action. Recently, while replying to Wedgwood and Ford [1924] I confirmed [Bezssonoff, 1924, 2] my first supposition: the blue reaction is not given by the vitamin C itself, but by an unstable radical, which is split off from it.

In the article published in 1922 [Bezssonoff, 1922], I gave an example of a liquid not containing vitamin C and giving the blue reaction (urine of an animal fed with substances rich in vitamin C), and also that of a liquid containing vitamin C and not giving the reaction (cow's milk). As I have since shown [Bezssonoff, 1924, 2] by introducing a very simple complemental condition for testing with my reagent, the absence of vitamin from urine as well as its presence in a milk can actually be certified. The liquid to be tested is heated in a test-tube (boiled for about 15 sec.) or maintained in boiling water for 5 to 10 minutes. After cooling, the coloured reaction obtained is compared with that given by the same liquid unheated. It is best to use a weakly acidified liquid. If vitamin C was present in the liquid, the coloration in the heated tube is much more intense than in the unheated tube. It can be intensified

as much as four times. If the unheated liquid gives no coloration, it appears in the heated tube (example cow's milk [Bezssonoff, 1924, 2]). On the other hand, the blue reaction given by a solution not containing vitamin C diminishes or completely disappears in the heated tube. Kay and Zilva can easily convince themselves that their preparation which did not give the blue reaction though containing vitamin C, would give this reaction if first submitted to a short heating of 10 minutes in a water-bath. The intensification will be greater if they use an acidified solution (5 % HCl).

Quite different is the case concerning the observations of the authors on yeasts. The extracts and preparations obtained from yeast give, with my reagent, a yellow-brown coloration [Bezssonoff, 1924, 1] or none at all. Plum juice [Bezssonoff, 1922] gives the same reaction and it is known [Osborne and Mendel, 1920; Hess and Unger, 1918] that this fruit, like yeast, is rich in vitamin B and does not contain vitamin C. But this brown-yellow reaction can only be obtained with these substances if the reagent used has been prepared from sufficiently purified crystals of the acid $(WO_3)_{17}.MoO_3.P_2O_5$, 24 H_2O. By using a mixture or badly purified crystals, the yellow-brown coloration is not obtained but a blue one. I convinced myself that the aqueous extract of brewer's top fermentation yeast gave an absolutely yellow-brown coloration with my pure reagent. This same yeast washed, dried and digested in water according to the procedure followed by Kay and Zilva gave a weak yellow-brown coloration and an abundant precipitate. After separating the precipitate, the colour of the liquid could no longer be distinguished. Unfortunately, I could not get the commercial preparation obtained from yeast named "marmite." But using an aqueous extract of a French commercial preparation of dried yeast, I obtained a brown-yellow reaction. In all the cases just mentioned, by using a reagent prepared from badly washed crystals or simply the mixture which is used to prepare the crystals for the reagent, instead of the yellow-brown coloration, a blue reaction is obtained. Simonet [Bezssonoff, 1924, 1], testing with my reagent a preparation from yeast rich in vitamin B, obtained a yellow-brown coloration. I must add that a specially purified preparation of vitamin B obtained from yeast by Seidell's method, tested in the latter's presence, gave a pale yellow coloration hardly visible. Recently, a similar preparation, which had been tested on animals (kindly given me by Nakamoura, G. Bertrand's laboratory, Pasteur Institute) used in 1/2 % aqueous solution to which 5 % HCl had been added, was heated on the autoclave at 120° during 45 minutes. The cooled liquid tested with a diluted reagent (0·7 % of crystals = 6 drops to 2 cc.) gave a slight flocculation and a *distinctly* yellow-brown coloration which was slowly absorbed by the precipitate.

In a note published in this *Journal*, but overlooked by the authors [Bezssonoff, 1923], I insisted upon the necessity of preparing the reagent from crystals and I gave a simple method for doing it and for verifying the sufficient degree of purity of the crystals obtained. I must add that in testing the degree of purity of the crystals obtained in this way, it is important to be convinced that

the yellow-brown reaction given by weak dilutions of pyrogallol is sufficiently stable, *i.e.* can be preserved at least for two hours without turning blue. This change of colour is produced very rapidly in the case of badly washed crystals.

In 1922 [Bezssonoff, 1922] when I said that it was better to use a reagent prepared from crystals, I did not insist enough on this point and I described my first method of preparing the reagent, which consisted in making a mixture (modification of Folin's mixture). In my reagent are present the three acids which were first introduced in a reagent by Folin, but it is necessary to lay stress on the chief differences which exist between Folin's typical reagent and mine. Folin [Folin and Denis, 1912] expressed the opinion that in this reagent the reacting substance must be very complex. According to Wu [1920], this substance consists of a mixture of two tungsto-phospho-molybdic acids: $(WO_3)_{13}.(MoO_3)_5.P_2O_5$ and $(WO_3)_{14}.(MoO_3)_4.P_2O_5$. My reagent contains only one phospho-molybdo-tungstic acid of this series. In this acid, the molybdenum is present in the smallest proportions that can exist and, as indicated by Wu, the reduction (blue coloration) is obtained with more facility if the proportion of molybdenum in the phospho-molybdo-tungstic acids is greater. Thus we can easily understand that when the acid present in my reagent is not used in a sufficient degree of purity, the presence of other acids containing a greater proportion of molybdenum gives a blue coloration when it ought not to be produced.

Conclusions.

1. A complementary condition for testing antiscorbutic liquids with Bezssonoff's reagent is given; it consists in shortly heating the liquid before testing it for 5 to 10 minutes in a water-bath in neutral or slightly acid medium.

(*a*) When the unheated liquid, though containing the vitamin C, does not give the reaction, it appears in the heated (and then cooled) liquid.

(*b*) The colour reaction already given in the unheated tube is distinctly intensified in the heated tube, when the liquid contains vitamin C. It diminishes or completely disappears when the liquid does not contain vitamin C.

2. The reagent must be prepared from crystals sufficiently purified and tested for their degree of purity [Bezssonoff, 1923].

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LI. INVESTIGATION ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS. PART V.

DIURNAL VARIATIONS IN THE PROTEIN NITROGEN OF RUNNER BEAN LEAVES.

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The object of the present research was to establish definitely whether there is a diurnal fall in the protein content of runner bean leaves at night.

In a former paper [Chibnall, 1923, 2] the author discussed at some length the available evidence concerning the diurnal changes in the total nitrogen content of foliage leaves, and concluded that there was undoubtedly a fall at night. In comparing the day and night samples of leaves three methods had been used to estimate the total nitrogen present, namely, those in which it was expressed as

A. Weight in terms of a certain number of leaves.

B. A percentage of the dry leaf weight.

C. A percentage of the fresh leaf weight.

It was further emphasised that method B might lead to an inaccurate and misleading interpretation of the diurnal change: that method A was unreliable unless some regard was paid to sampling errors: that method C would give the most accurate interpretation of the true diurnal change; conclusions amply justified by the results of the present research.

The above criticisms will apply with equal force to any determination concerning the diurnal variation in the protein nitrogen content of the leaf; in the following paragraphs the work of earlier investigators is discussed in terms of them.

Schulze and Schütz [1909] made a thorough investigation into the seasonal and diurnal changes in the leaves of the box elder (*Acer negundo*). The leaves were picked from two trees 15–20 years old, and those in each sample were chosen as far as possible of equal weight and leaf area. The evening leaves were picked between 6 p.m. and 6.30 p.m., those on the following morning between 5 a.m. and 6.30 a.m., depending on the season. The leaves were

rapidly dried at 90° (which may have brought about slight proteolysis), and protein nitrogen determined by Stutzer's method. They themselves interpreted their results by method A; it is possible from the data they supply to recalculate those by method C. The results by both methods are given in Table I.

Table I. *Showing the change in the amount of protein nitrogen in the leaves of* Acer negundo *at night, expressed as a percentage of the day value. (Schulze and Schütz.)*

	Change	
Date picked	Method A %	Method C %
7 May	−10·20	−9·18
6 June	−16·62	−6·88
5 July	− 8·29	−3·60
2 August	+ 2·51	+1·98
25 Sept.	+ 4·77	+4·73

In the absence of sampling errors the present author prefers to interpret the results in terms of method C, which clearly shows that there is a decided fall in the protein nitrogen at night during the period of physiological activity of the trees. Further, the figures for the water-soluble nitrogen, which remain more or less unchanged, show that the lost protein nitrogen has been translocated away from the leaf. When the leaves reach maturity, and growth has more or less ceased, the withdrawal is no longer apparent. The somewhat large rise in the protein nitrogen on the night of 25 Sept. may be a misleading interpretation, since the leaves had then already begun to fall, indicating more or less rapid loss of water due to dehydration.

Suzuki [1897] attempted to find the diurnal change in the leaves of several plants. He expressed his results in terms of method A, but did not take into account sampling errors, nor were sufficient data given for results by method C to be calculated. The diurnal changes occurring between 6 a.m. and 6 p.m. were compared. The leaves were air-dried at an unstated temperature, so that proteolysis was possible. Protein nitrogen was determined by Stutzer's method. His results are given in Table II, the figures in brackets after each plant denoting the number of leaves per sample used.

Table II. *Showing the change in the protein nitrogen in the leaves of various plants at night, expressed as a percentage of the day value. (Suzuki.)*

Date picked	Name of plant	Change by method A %
—	*Wistaria brachybotrys* (400)	−16·6
17 Sept.	*Phaseolus mungo* (200)	− 9·9
29 Oct.	„ *vulgaris* (50)	−10·9
5 June	*Solanum tuberosum* (220)	− 5·1
18 Sept.	*Batatus edulis* (100)	− 4·2
26 Sept.	*Polygonum fagopyrum* (450)	− 7·5

Suzuki gives no indication as to the physiological period at which his leaves were picked, but as most annuals show continuous growth until the death of

the plant, it is probable that there is a continuous call on the leaves for nitrogenous compounds. Though sampling errors were not taken into account Suzuki used in most cases a large number of leaves, so that his results, which are in general agreement with the better experimental data of Schulze and Schütz, are worthy of consideration.

Pigorini [1914] studied the diurnal variations in the leaves of the mulberry (*Morus alba* var. *Limoncina*). Thirty-eight leaves were picked at 5 p.m. 7 Sept. and 39 leaves at 5 a.m. the following morning. After drying, the protein nitrogen was determined by a modification of Stutzer's method. His result by method C shows a fall in the protein nitrogen content at night of 11·5 %, the major part of this lost nitrogen having been translocated away from the leaf.

The above results seem to indicate that during periods of physiological activity in the higher plants protein decomposition takes place in the leaf at night. The figures quoted above, however, must be accepted with caution, and in no case as quantitative, since the protein nitrogen has been estimated by Stutzer's method. This method is conventional only, and assumes that after the dried, ground up leaves are boiled with Stutzer's reagent, all the nitrogen remaining insoluble is to be regarded as protein nitrogen. Further, unless the drying of the leaves is very rapid, there will be loss of protein due to autolysis [Chibnall, 1922].

All the results quoted above suffer from the disadvantage that the diurnal change has been taken on one sample of day and night leaves only, so that errors due to sampling and individual differences are uncontrolled. In the present research nine diurnal samples of runner bean leaves have been taken, thereby allowing the degree of probability of the recorded results to be calculated.

General Experimental Methods.

Materials used. The plant used was the runner bean (Scarlet Champion), *Phaseolus vulgaris* var. *multiflorus*. The seeds were planted during the first week of April, 1923, in unmanured ground at the Physic Garden, Chelsea, following beans of similar species. Growth was delayed by cold weather in May, but by mid-August, when the experiments to be described in this paper were commenced, the plants were fully grown and in process of pod-formation.

Methods of sampling. The leaves of *P. vulgaris* var. *multiflorus* are pinnate trifoliate. Those used in the present experiments were fully grown, and were chosen from the upper parts of the plant, thereby ensuring that their surfaces were free from soil contamination.

A day or so before the experiment was to be performed the plants were carefully examined, and three batches each of about 100 leaves, which appeared to have basal and opposite leaflets of equal development, were selected. These were marked with coloured paint on the terminal leaflet to aid future identification.

At 8 p.m. on the night of the experiment, just before sunset, one of every

pair of basal and opposite leaflets was removed without any attached petiole. As the leaflets were picked they were placed one above the other in a closed box so as to reduce respiration to a minimum, and as soon as each batch was collected the total weight was taken. The leaflets were then counted. The time required for the picking and weighing was 20 minutes, so that all three batches could be dealt with in an hour.

At 6 a.m. the following morning, about an hour and a half after sunrise, the corresponding leaflets were detached and treated in a similar way.

Methods of manipulation (separation of vacuole material from the leaf cells). The ether method recently described by the author [Chibnall, 1923, 1] was used[1]. In that paper it was shown that the leaf cell constituents of spinach were separated into two portions, consisting of

(*a*) An extract containing *all* the vacuole content, with possibly some constituents of the cytoplasm, which, at the time of death, have either passed into the vacuole, or have been subsequently removed from the cytoplasm by the dilute acid (0·002 *N*) used for washing the residue.

(*b*) A residue consisting of cell wall material, together with the cell cytoplasm, from which some of the constituents may have been removed, as per (*a*) above. The cytoplasmic material could afterwards be extracted by grinding, and was found to consist of protein (N = 15 % ash free) and substances soluble in alcohol and ether.

Preliminary experiments with bean leaves showed that a similar separation could be readily effected. Immediately after weighing and counting, the batch of leaves was placed in a large beaker, covered with ether for one minute, and the ether drained off. The plasmolysed leaves[2] were then wrapped in a small piece of stout, well washed, filter cloth, and subjected to slowly increasing pressure in the Buchner press. The major part of the vacuole material, a clear amber-coloured fluid, was readily expressed. The filter cloth and contents were removed from the press, placed in a shallow enamelled iron tray, the cloth opened, and the exposed mass of flattened leaves allowed to absorb 0·002 *N* HCl (as with spinach, acid of this strength, in place of distilled water, was necessary to prevent the partial solution of the cytoplasmic proteins). The cloth was then again closed and pressure applied as before. This operation was repeated four times, so that the material was washed five times in all. All six extracts were combined, and the total volume measured.

The total time taken to extract each batch was 40 minutes; too short for post-mortem autolysis to have brought about any marked chemical change. How complete was the extraction is illustrated by Table IV (p. 392).

[1] In that paper the term "protoplasmic material" was meant to embrace the chloroplasts and other possible inclusions. "Cytoplasmic material" expresses this without ambiguity: it will, therefore, be used in future in place of the former term.

[2] The ether causes the cell protoplast to contract, the vacuole material passing freely through it. It is really false plasmolysis, since true plasmolysis, according to the definition of de Vries, implies the passage of water, and not dissolved vacuole material, through the protoplast.

Distribution of solids.

Vacuole material. An aliquot part of the vacuole extract was run into a tared beaker and evaporated to dryness on a water-bath. The beaker was then dried at 108° for 24 hours, cooled, and weighed.

Cell wall and cytoplasmic material. The leaf residue from the press was removed quantitatively from the filter cloth, and dried at 108° in an oven for 24 hours.

Distribution of protein N and non-protein N.

Protein N. As stated above, the residue from the press contains the cell wall and washed cytoplasmic material. The cell wall material contains no appreciable amount of N. The washed cytoplasmic material consists of protein together with substances soluble in alcohol and ether. Some of these latter, such as chlorophyll, phosphatides, etc. contain N, but the total amount, compared with the protein N present, is small, and not subject to diurnal variation. Any appreciable diurnal change of N in the pressed residue, then, can be ascribed to a change in the total amount of protein present.

The vacuole extract also contains protein. This was coagulated by boiling, filtered off and washed free from extract with water. The filter paper with the retained protein was then transferred to a Kjeldahl flask. Total protein N was taken as the sum of the N in the pressed residue and the coagulum obtained by boiling the extract.

Non-protein N. This was determined in the vacuole extract (after boiling to coagulate and remove protein) by Kjeldahl's method.

Table III *Illustrating the separation of the protein N and non-protein N in a sample of* 149 *g. of leaves.*

Phaseolus vulgaris *var.* multiflorus.

		Percentage of total leaf N	N as a percentage of the fresh leaf weight
1st extract		4·64	0·0314
2nd ,,		1·35	0·0092
3rd ,,		0·89	0·0060
4th ,,		0·56	0·0038
5th ,,		0·33	0·0022
6th ,,		0·33	0·0022
Total non-protein N		8·10	0·0548
Total protein N	In coagulate	1·60	0·0106
	In residue	90·30	0·6084
Total leaf N		100·00	0·6738

Results.

The results of a series of three experiments, giving the diurnal variations in nine samples of leaves, are recorded in Tables IV–VIII. Table III is given to illustrate how complete is the separation of the protein N and non-protein N by the method described above. In this case each successive extract from the press was boiled to coagulate the protein, filtered and the N in it determined. It will

Table IV. *Showing details of samples used.*

Phaseolus vulgaris *var.* multiflorus.

Date picked	Number of leaves in sample	Weight 8 p.m. g.	Weight 6 a.m. g.	Change
14/15 Aug.	90	126·8	127·1	+0·3
,, ,,	96	133·6	130·4	−3·2
,, ,,	101	125·5	117·2	−8·3
20/21 ,,	93	110·1	108·8	−1·3
,, ,,	96	115·6	108·0	−7·6
,, ,,	115	116·1	116·3	+0·2
25/26 ,,	94	138·2	135·3	−2·9
,, ,,	91	111·5	114·2	+2·7
,, ,,	80	89·9	90·5	+0·6

Table V. *Variation of leaf total solids at night.*

Phaseolus vulgaris *var.* multiflorus.

(*Solids expressed as a percentage of the fresh leaf weight; change as a percentage of the* 8 *p.m. value.*)

Date picked	Cell wall + cytoplasmic material 8 p.m. %	6 a.m. %	Change %	Vacuole material 8 p.m. %	6 a.m. %	Change %	Total solids 8 p.m. %	6 a.m. %	Change %
14/15 Aug.	9·70	9·53	−1·7	4·41	4·45	0·0	14·10	14·00	−0·1
,, ,,	9·50	9·50	0·0	4·82	4·86	0·0	14·34	14·35	0·0
,, ,,	9·63	9·68	+0·5	4·41	4·70	+ 6	14·04	14·38	+0·2
20/21 ,,	9·80	9·75	−0·5	4·67	4·45	− 4	14·46	14·20	−0·2
,, ,,	10·31	9·96	−3·5	4·34	4·67	+ 6	14·67	14·63	0·0
,, ,,	10·35	9·85	−5·0	4·05	4·54	+11	14·40	14·40	0·0
25/26 ,,	10·60	10·27	−3·1	3·96	4·53	+13	14·56	14·79	+0·2
,, ,,	10·03	9·45	−5·8	3·67	4·09	+10	13·70	13·54	−0·1
,, ,,	10·63	10·17	−4·3	3·60	4·28	+17	14·23	14·44	+0·1
		Mean	−2·6 ±0·5		Mean	+ 6 ± 1·3		Mean	0·0

Table VI. *Variation of leaf total solids at night.*

Phaseolus vulgaris *var.* multiflorus.

(*Solids expressed as a weight per* 100 *leaves; change as a percentage of the* 8 *p.m. value.*)

Date picked	Cell wall + cytoplasmic material 8 p.m. g.	6 a.m. g.	Change %	Vacuole material 8 p.m. g.	6 a.m. g.	Change %	Total solids 8 p.m. g.	6 a.m. g.	Change %
14/15 Aug.	13·5	13·5	0·0	6·14	6·29	− 2	19·65	19·76	+0·1
,, ,,	13·2	12·9	− 2·3	6·70	6·60	− 1	19·90	19·50	−0·2
,, ,,	12·0	11·3	− 5·8	5·44	5·47	0	17·44	16·74	−0·4
20/21 ,,	11·6	11·4	− 1·8	5·55	5·20	− 5	17·13	16·61	−0·3
,, ,,	12·4	11·2	−10·0	5·23	5·26	0	17·67	16·46	−0·7
,, ,,	10·4	10·0	− 4·0	4·09	4·60	+12	14·54	14·55	0·0
25/26 ,,	15·57	14·78	− 5·3	5·84	6·52	+12	21·40	21·30	−0·0
,, ,,	12·28	11·86	− 3·3	4·50	5·13	+13	16·80	17·00	+0·2
,, ,,	11·95	11·50	− 4·1	4·05	4·84	+20	16·00	16·34	+0·2
		Mean	− 4·1 ± 0·7		Mean	+ 8·0 ± 2·0		Mean	0·0

Table VII. *Variation of leaf protein N, non-protein N and total N at night.*

Phaseolus vulgaris *var.* multiflorus.

(*Nitrogen expressed as a percentage of the fresh leaf weight; change as a percentage of the 8 p.m. value.*)

Date picked	Protein N			Non-protein N			Total N		
	8 p.m. %	6 a.m. %	Change %	8 p.m. %	6 a.m. %	Change %	8 p.m. %	6 a.m. %	Change %
14/15 Aug.	0·576	0·562	−2·7	0·077	0·070	− 9	0·654	0·632	−3·4
,, ,,	0·550	0·546	−0·7	0·074	0·065	−12	0·623	0·611	−1·9
,, ,,	0·585	0·579	−1·1	0·076	0·071	− 7	0·661	0·650	−1·6
20/21 ,,	0·565	0·550	−2·7	0·066	0·059	−10	0·630	0·607	−3·7
,, ,,	0·608	0·592	−2·7	0·070	0·066	− 6	0·678	0·659	−2·8
,, ,,	0·589	0·575	−2·4	0·067	0·063	− 6	0·656	0·639	−2·6
25/26 ,,	0·623	0·614	−1·4	0·058	0·053	− 8	0·681	0·666	−2·2
,, ,,	0·595	0·584	−1·8	0·065	0·055	−15	0·660	0·639	−3·2
,, ,,	0·639	0·635	−0·6	0·067	0·061	− 9	0·706	0·696	−1·4
		Mean	−1·8		Mean	− 9·0		Mean	−2·5
			±0·2			± 0·6			±0·2

Table VIII. *Variation of leaf protein N, non-protein N and total N at night.*

Phaseolus vulgaris *var.* multiflorus.

(*Nitrogen expressed as a weight per 100 leaves; change as a percentage of the 8 p.m. value.*)

Date picked	Protein N			Non-protein N			Total N		
	8 p.m. g.	6 a.m. g.	Change %	8 p.m. g.	6 a.m. g.	Change %	8 p.m. g.	6 a.m. g.	Change %
14/15 Aug.	0·803	0·793	−1·2	0·108	0·099	− 8	0·911	0·892	−2·1
,, ,,	0·765	0·742	−3·0	0·102	0·089	−13	0·892	0·831	−4·2
,, ,,	0·728	0·672	−7·6	0·094	0·082	−13	0·822	0·754	−8·3
20/21 ,,	0·669	0·643	−3·9	0·078	0·067	−14	0·747	0·711	−4·8
,, ,,	0·732	0·667	−7·6	0·085	0·074	−13	0·817	0·741	−9·3
,, ,,	0·595	0·582	−2·2	0·068	0·064	− 6	0·663	0·647	−2·5
25/26 ,,	0·918	0·884	−3·7	0·085	0·074	−13	1·001	0·958	−4·3
,, ,,	0·729	0·733	+0·6	0·079	0·069	−12	0·808	0·802	−0·7
,, ,,	0·718	0·719	+0·1	0·075	0·069	− 8	0·793	0·788	−0·6
		Mean	−3·3		Mean	−11·0		Mean	−4·1
			±0·6			± 0·7			±0·7

be seen that N in the last two extracts is only 0·0022 % of the fresh leaf weight, whereas the mean change in the protein N at night, as given by Table VII, is 0·010 % of the fresh leaf weight. Since in the experiments illustrated by Tables IV–VIII care was taken to standardise the conditions under which the non-protein N was extracted, it is justifiable to assume that the changes in protein N recorded are not due to differences in the degree of extraction of the water-soluble products from the leaf cells.

In Table IV two out of the nine diurnal samples taken show a difference of 6 % in weight, far greater than one could assign to translocation away from the leaf at night, and obviously due to different development of the basal and opposite leaflets. If comparison is made between Tables V and VI, and between VII and VIII it will be seen that Tables V and VII, based on method C are more reliable than Tables VI and VIII, based on method A, which confirms the conclusion given in the early part of this paper, that method A is unreliable unless sufficient samples are taken to allow probable errors to be calculated.

Discussion of Results.

Diurnal variations in the solids (Tables V and VI). Assuming that the cell wall material remains unchanged, it will be seen that there is a decrease in the cytoplasmic material at night, with a corresponding increase of soluble vacuole products. This is probably due to the breaking down of starch and protein. It is surprising that the total solids show no change at night. Since pods were forming at the time when these experiments were made there must have been some call on the leaves for carbohydrate. Assimilation in the weak light between sunrise and 6 a.m. ($1\frac{1}{2}$ hours) making up for losses at night, or the translocation of sugar taking place during the day, may possibly explain this.

Protein N and non-protein N. Table VII shows a fall in the protein N at night of 1·8 %, with a probable error of only 0·2 %. It can therefore be taken as an established fact that protein decomposition takes place in the leaf cells at night. Further, although it is not shown in the tables, the loss of protein is from the cytoplasm, and not from the small traces normally present in the vacuole. The non-protein N also shows a definite decrease of 9·0 % with a probable error of only 0·6 %. Both "ammonia N" and "amide N of asparagine" remained unchanged at 0·001 % and 0·005 % of the fresh leaf weight respectively, so that the products of the protein decomposition have undoubtedly been translocated to other parts of the plant. The establishment of these facts is of undoubted physiological importance, but further discussion is held over until the next paper, wherein the nature of these protein decomposition products is investigated.

Summary.

By properly controlled experiments it is shown that there is a definite fall n the protein content of bean leaves at night, due to the breaking down of the cytoplasmic material. The nitrogenous products of this protein decomposition are translocated away from the leaf.

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LII. INVESTIGATIONS ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS. PART VI.

THE RÔLE OF ASPARAGINE IN THE METABOLISM OF THE MATURE PLANT.

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Introduction.

Our knowledge of the nitrogenous metabolism of the higher plants is still in a very incomplete state. Such as we have is based more or less entirely on experiments made with germinating seedlings. Obviously the metabolic changes thus shown are only of limited application and cannot be applied to the mature plant, since the nitrogenous metabolism of seedlings concerns mainly the decomposition of already existing protein stored up in the cotyledons and its regeneration at the growing points. As soon as this reserve is exhausted, as it usually is after the 14th–20th day, the plant metabolism develops so as to include the synthesis of protein and other organic nitrogenous materials from the simple substances, such as nitrates, which the root-system can extract from the soil. The focus of metabolic activity is then transferred to the leaves, which become the seat of protein, as well as of carbohydrate, synthesis. For an adequate knowledge of the metabolic processes occurring in the mature plant then, it is of the first importance that light should be thrown on the nitrogenous metabolism in the leaf.

The function of the protein in the leaf, and the nature of the compounds with which the leaf supplies the other parts of the plant to make up for losses due to living processes and for growth has received but small attention in the past.

Borodin [1878], using microchemical methods, stated that the disappearance of protein in leaves was accompanied by the appearance of asparagine. Schulze [1880], who cut young shoots from the birch and placed them with their cut ends in water for 10–12 days, found an accumulation of asparagine in the leaflets. Later Schulze and Bosshard [1885] repeated the experiment with young oat plants, and found an increase of asparagine at the expense of protein (see Table I).

Table I. *Showing the protein decomposition in young oat plants kept in a dark room for eight days. (Schulze and Bosshard.)*

	In percentages of the dry weight	
	Fresh plants, taken from field and analysed direct	Plants cut and left with stems in water in a dark room for 8 days
Total N	4·12	4·50
Protein N (Stutzer)	3·51	1·46
Asparagine N (Sachsse)	0·15	1·69
Other N (diff.)	0·46	1·35

Butkewitsch [1908], enlarging on this early work of Schulze's, showed that the increase of asparagine corresponded roughly to the decrease of protein and pointed out that this was analogous to the decomposition of the cotyledon reserve proteins in the germinating seedling.

In the above-quoted experiments the root-system had been detached, but even so the normal translocation of substances from the leaves to other parts of the plant and *vice versa* was still possible. Therefore, the accumulation of asparagine cannot be correlated definitely with the metabolism of the leaf proteins.

Only one experiment so far has been made with leaves completely detached from the plant, so that any accumulation of substances can be ascribed definitely to metabolic processes in the leaf itself. Suzuki [1897] showed that during the night there was a decrease in the leaf protein [for criticism of this statement see the preceding paper, Chibnall, 1924], and, since the amounts of asparagine and what he referred to as "amido" compounds, did not increase, he concluded that the protein had been resolved into these substances, which were then translocated away from the leaf. Negative results of this nature, however, unsupported by further experimental data, cannot be considered as sufficient evidence to support such a far-reaching conclusion.

In the preceding paper [Chibnall, 1924] the author has shown, by properly controlled experiments, that there is undoubtedly a fall in the protein content of runner bean leaves at night; also that the products of this decomposition are not stored up in the leaf, but translocated. In this present paper experiments have been devised so as to throw light on the chemical nature of these leaf protein decomposition products. It is found that about one-half of the leaf protein nitrogen has passed into asparagine, and about one-quarter into other amino bodies of undetermined composition. Asparagine, then, is one of the chief products of the nitrogen metabolism of the mature leaf. The conclusion is reached that this substance is, in the mature plant, one, and probably the most important, of the means whereby nitrogen in a form suitable for easy resynthesis of protein is conveyed from one part of the mature plant to another. This has already been shown to be the case in germinating seedlings.

General Statement of the Problem to be Investigated.

In the present paper the author offers no evidence concerning protein synthesis in leaves, but is concerned only with the composition and fate of its decomposition products.

The problem was attacked in the following way. There is evidence, collected and confirmed in the preceding paper [Chibnall, 1924], that the total amount of protein present in the leaf undergoes a decided diminution during the night, and that, whatever the nature of its decomposition products, these are not stored up in the leaf but translocated, since the water-soluble nitrogen in the leaf shows very little diurnal change.

The object of the present research was to devise means whereby these decomposition products could be submitted to chemical analysis.

The method adopted was to cut from the plant leaves with long petioles and allow them to remain with their petioles in water for four or five days. The leaves remained fresh and turgid throughout, so that respiration, though probably not normal, was not interfered with [compare Spoehr and McGee, 1923]. Although the conditions thus produced are artificial they have the advantage that translocation to and away from the leaf is eliminated. Therefore the products of nitrogenous metabolism, which normally appear to migrate, accumulate, and so become available for chemical analysis.

General Experimental Methods.

Materials used. The plant used was the runner bean (Scarlet Champion), *Phaseolus vulgaris* var. *multiflorus*. The seeds were planted about the end of April, 1922, in unmanured ground at the Physic Garden, Chelsea, following plants of the same species the previous year. Growth was normal, and during August and early September, when the samples of leaves used during the present research were picked, the plants were in process of pod formation. The one experiment made during 1923 was with leaves from plants used in the research described in the preceding paper [Chibnall, 1924]. Flower buds were just about to form at the time of picking.

Description of experiments. Large batches of mature leaves with long petioles were cut from the plant. The ends of the petioles were then at once cut under water, and bunches of about 30 leaves were placed in glass jugs filled with tap water so that the petioles were immersed. The leaves themselves were well above the water and both their upper and lower surfaces were freely exposed to the air, so that respiration was not interfered with.

They remained thus for four or five days in either strong diffused daylight or in the dark, depending on the conditions of the experiment being performed. The tap water in the jugs was changed daily, and about $\frac{1}{4}''$ from the end of each petiole was also removed daily, so as to be sure that bacterial contamination did not interfere with the free supply of water through the petiole to the leaf.

At the end of the prescribed time the leaves were withdrawn from the jugs, care being taken not to wet the leaf surfaces, the petioles removed, and the leaves subjected to the analysis described below.

At the same time that the leaves for the above experiments were removed from the plant, control batches of leaves were analysed directly after cutting, so that the change in the nitrogenous products of the leaves placed with their petioles in water could be followed. Details of samples used are given in Table II.

Table II. *Giving details of the samples of bean leaves used.*

Phaseolus vulgaris *var.* multiflorus.

Date picked (10–12 a.m.)	Number of days with petioles in water	Total weight of leaves in sample (g.)
5 August, 1922	4 in light	1200
8 ,, ,,	0	2010
12 ,, ,,	4 in dark	2400
23 ,, ,,	0	5240
25 ,, ,,	5 in light	2740
26 ,, ,,	5 in dark	1870
7 Sept. ,,	4 in light	2020
11 ,, ,,	0	400
12 July, 1923	0	258
12 ,, ,,	4 in dim light	132

Separation of water-soluble products from the leaves. The fresh leaves, free from all extraneous moisture, were first weighed, and then a sample of about 200 g. removed for determinations of dry weight and total nitrogen.

The water-soluble products were then extracted from the remainder by the earlier ether-water method of Chibnall and Schryver [1921]. The extract from the press was boiled to coagulate the proteins, and subsequently filtered. The residue from the press was then extracted three times successively with boiling water, the mixture, after cooling, being pressed out in the tincture press. By this means the major part of the water-soluble products are obtained in aqueous solution free from all protein and colloidal material. The object of the present research was qualitative rather than quantitative, and in no case is it certain that the water-soluble products were quantitatively removed. The amount remaining in the pressed residues was, however, very small.

The above practical details refer to samples of leaves picked in 1922. In the one experiment made in 1923 the newer method of extraction, described in the previous paper [Chibnall, 1924], which gives a quantitative result, was used.

Total protein and non-protein nitrogen. From previous work on the leaves of the runner bean [Chibnall, 1922] it is clear that the water-soluble nitrogen, as separated above, is non-protein nitrogen (proteoses may be present). The nitrogen of the pressed residues and of the coagulum obtained by boiling the extract, is mostly protein nitrogen, for small amounts due to chlorophyll, etc. can be neglected in this present research.

Distribution of nitrogen in the water-soluble products. Total N, ammonia N, and "amide N of asparagine" were determined by the methods described in a former paper [Chibnall, 1922]. After removal of the ammonia set free from the asparagine by the 4 % HCl hydrolysis (according to Sachsse's method), the excess of magnesia was dissolved by adding a small excess of glacial acetic acid. This solution was then made up to a standard volume, and free amino N determined by the method of Van Slyke. Urea was determined by the urease method of Folin and Wu, but it was present only in minute amounts, and did not increase in the leaves placed with their petioles in water.

Results are given in Tables III and IV.

Isolation of asparagine. It is to be remembered that Sachsse's method gives only the nitrogen in the acid amide linkage $CO–NH_2$ and is not necessarily evidence of the presence of asparagine. Actual evidence that asparagine was present was obtained by the crystallisation method of Schulze and Winterstein [1910, 1, 2]. The plant extract was treated with neutral lead acetate in slight excess and allowed to stand 24 hours. The resulting precipitate was then centrifuged off and washed. The residual liquid and washings were next treated with mercuric nitrate until no further precipitation occurred, the solution being kept nearly neutral to litmus by means of sodium hydroxide. After standing 24 hours the precipitate was centrifuged off, washed and decomposed with H_2S. The precipitate of HgS was centrifuged off, and the clear liquid made neutral by a few drops of strong ammonia. It was then evaporated on the water-bath to a small bulk, a drop or two of ammonia being added occasionally to keep the reaction neutral. The concentrated solution was then allowed to crystallise slowly in a vacuum desiccator. 500 cc. of the extract made 7th September were first treated in this way. From preliminary analyses this solution contained 2·28 g. of N and 0·294 g. of "amide N" (Sachsse). The distribution of "amide N" (Sachsse) was followed in the separation, and 64·2 % of it was carried down in the mercury precipitate. The yield of recrystallised asparagine ($C_4H_8O_3N_2.H_2O$) was 0·75 g. or 30 % of that given by Sachsse's method. It gave the following analysis.

	Found	Calculated for $C_4H_8O_3N_2.H_2O$
Amide N (Sachsse)	9·01 %	9·34 %
Amino N (Van Slyke)	9·51	9·34

Since portions of the various solutions had been withdrawn for analysis this yield was not a maximum. Therefore a further isolation was made from 660 cc. of the extract made 12th August containing 3·20 g. of total N and 0·457 g. of "amide N" (Sachsse). The preparation was recrystallised twice from hot water. The final product, after drying for some days in a vacuum desiccator, weighed 1·958 g. It gave the following analysis:

	Found	Calculated for $C_4H_8O_3N_2.H_2O$
Water of crystallisation (by heating to 108° for 1½ hours)	12·12 %	12·00 %
Total N (Kjeldahl)	18·44	18·68
Amide N (Sachsse)	9·16	9·34
Amino N (Van Slyke) ...	9·48	9·34

The asparagine remaining in the mother liquids from the second and third recrystallisations was converted into the Cu salt by boiling with $Cu(OH)_2$, filtering and allowing to cool. The copper salt gave on analysis 19·59 % of Cu; calculated for asparagine 19·50 %. The gross yield of pure asparagine ($C_4H_8O_3N_2.H_2O$) was 2·394 g. or 48·8 % of that indicated by Sachsse's method. No trace of glutamine was found. As the previous isolation from the extract made 7th September had shown that only 60–70 % of the "amide N" (Sachsse) was precipitated by the mercuric nitrate the yield stated above can be regarded as satisfactory for that fraction. An attempt was also made to isolate asparagine from one of the control plant extracts. 1375 cc. of the extract made 23rd August containing 3·20 g. of total N and 0·176 g. of "amide N" (Sachsse) were submitted to the above treatment, but no crystals of asparagine were obtained.

The application of this method for the isolation of asparagine and glutamine from plants has been chiefly employed by Schulze and his co-workers at Zürich. With germinating seedlings of lupins, in which asparagine makes up nearly two-thirds of the total water-soluble products, Schulze [1880] obtained a yield corresponding very closely with that given by Sachsse's method. When, however, he used the green parts of mature plants his yield of crystalline asparagine was greatly reduced. Thus Schulze and Bosshard [1885], from 1 kilo. of fresh oat plants, were unable to isolate any asparagine, though Sachsse's method indicated 0·27 g. of "amide N." When similar plants had been left with cut stems placed in water for eight days, thereby showing a tenfold increase of "amide N" by Sachsse, they obtained only a 20 % yield of crystalline asparagine. Butkewitsch [1908], who repeated this work of Schulze's, was more successful, and obtained from the plants left with their cut stems in water yields of crystalline asparagine equal to 40–45 % of that shown by Sachsse, a result similar to the present author's quoted above.

As to the nature of the remaining 30–40 % of "amide N" (Sachsse) in the bean extract of 12th August described above, there is, of course, no direct evidence. That part of it at least is derived from asparagine is probable, since it is hardly to be supposed that a complete separation of one single substance can be carried out by the method outlined above from such a complex mixture as the water-extract of a leaf.

As far as is known, no other substances that have been isolated from plants are capable of yielding ammonia when submitted to such mild hydrolysis. Urea, and, as a general class, hydantoins and the alloxan series are stable under this treatment. But it must not be forgotten that, as will be shown later, asparagine is undoubtedly the most important and most abundant product of protein metabolism in the leaf; consequently simple polypeptides containing this amino-acid may also be present. Thus Fischer has prepared several dipeptides containing asparagine, whilst Thierfelder and von Cramm [1919] have recently synthesised several polypeptides of glycine, alanine and glutamine. Such bodies have not yet been isolated from the plant, but the possibility

Table III. *Showing the increases in the various forms of non-protein N in the leaves placed with their petioles in water.*

Phaseolus vulgaris *var.* multiflorus.

(In percentages of total leaf N.)

Date picked	No. of days with petioles in water	Total non-protein N	Ammonia N	Amide N (Sachsse)	Free-amino N
8 August, 1922	0	13·54	0·28	0·64	6·00
5 ,,	4 in light	28·43	0·47	4·42	14·18
	Increase	14·89	0·19	3·78	8·18
8 ,,	0	13·54	0·28	0·64	6·00
12 ,,	4 in dark	31·70	0·45	4·53	14·07
	Increase	18·16	0·17	3·89	8·07
23 ,,	0	16·50	0·30	0·89	6·81
25 ,,	5 in light	29·84	0·37	4·46	15·77
	Increase	13·34	0·07	3·57	8·96
23 ,,	0	16·50	0·34	0·89	6·81
26 ,,	5 in dark	35·14	0·57	5·85	15·82
	Increase	18·64	0·23	4·96	9·01
11 Sept. 1922	0	16·75	0·46	1·06	7·40
7 ,,	4 in light	31·60	0·65	4·08	14·27
	Increase	14·85	0·19	3·02	6·87
12 July, 1923	0	14·05	0·30	0·75	—
12 ,,	4 in light	37·74	1·01	5·63	—
	Increase	23·69	0·71	4·88	—

Table IV. *Showing the increases in the various forms of non-protein N in the leaves placed with their petioles in water.*

Phaseolus vulgaris *var.* multiflorus.

(In percentages of total non-protein N.)

Date picked	No. of days with petioles in water	Total non-protein N	Ammonia N	Amide N (Sachsse)	Free-amino N
8 August, 1922	0	13·54	2·00	4·72	44·30
5 ,,	4 in light	28·43	1·65	15·55	49·85
	Increase	14·89	−0·35	10·83	5·55
8 ,,	0	13·54	2·00	4·72	44·30
12 ,,	4 in dark	31·70	1·42	14·29	44·37
	Increase	18·16	−0·58	9·57	—
23 ,,	0	16·50	2·00	5·98	41·30
25 ,,	5 in light	29·84	1·24	14·60	50·27
	Increase	13·34	−0·76	8·62	8·97
23 ,,	0	16·50	2·00	5·98	41·30
26 ,,	5 in dark	35·14	1·62	16·65	45·00
	Increase	18·64	−0·38	10·67	3·70
11 Sept. 1922	0	16·78	2·74	6·32	44·10
7 ,,	4 in light	31·60	2·06	12·87	45·16
	Increase	14·82	−0·68	6·55	1·06
12 July, 1923	0	14·05	2·16	5·30	—
12 ,,	4 in light	37·74	2·79	13·85	—
	Increase	23·69	0·63	8·55	—

of their accounting for part of the "amide N" (Sachsse) must not be overlooked.

Until further experimental evidence is available then, only 60–70 % of the "amide N" (Sachsse) obtained in the present bean extracts can be safely ascribed to asparagine.

Discussion of Results.

The results given in Tables III and IV can be summarised as follows:

(1) In the case of the leaves placed with their petioles in water there is, in terms of total leaf N, a large increase in non-protein N, with a corresponding decrease in the protein N.

(2) This increase is accounted for in large part by asparagine and free amino nitrogen.

(3) In terms of total water-soluble N the only increase is in the "amide N" (Sachsse); the concentration of the free amino N remaining more or less unchanged.

It is clear that one of the main products of the protein oxidation is ammonia. Free ammonia, however, is toxic to plants, so that it is probably removed by the formation of asparagine, which, even in high concentration, is innocuous to plants.

It must be remembered, however, that in the case of the leaves placed with their petioles in water conditions are artificial. As Pfeffer rightly emphasises [1897, p. 443] a substance that normally remains intact may be drawn into metabolism when the plant is starved, or the external conditions are altered in some way. Consequently the protein decomposition observed in the leaves that have had their petioles placed in water may be due not to the normal course of nitrogenous metabolism in the leaf but to abnormal conditions, chief among them being carbohydrate deficiency. That a continuous production of CO_2 is necessary in such leaves, even when kept in the dark, follows from some experiments of Spoehr and McGee [1923], who showed that although respiration decreases, it still remains active. Therefore, the possibility of the decomposition of protein and the production of CO_2 therefrom must not be overlooked; such a phenomenon is well known in animal metabolism.

Two facts, however, seem to indicate that this disappearance of protein is due, at any rate in part, to the normal course of protein metabolism in the leaf. In the first place the increase of total protein observed in leaves during the day, followed by a decrease at night [see Chibnall, 1924], indicates that protein decomposition in the leaf goes on continuously, and is only masked during the day by the fact that the rate of protein synthesis exceeds that of protein degradation. In the second place there is, in the case of the leaves kept with their petioles in water, a continuous and more or less equivalent fall both in the protein of those kept during the daytime in strongly diffused daylight, and in the protein of those kept continuously in the dark. As protein

synthesis in both these cases must be negligible, owing to the low concentration of nitrates and ammonium salts in the leaves when picked from the plants, it may be inferred that light has had no direct influence on the protein degradation. The disappearance of protein in these leaves, then, may not be due entirely to "starvation," but in part at least, due to the normal metabolism of the living cells. Table III, and the statements given earlier under general experimental methods, show that at least 35–40 % of the N from the protein undergoing degradation changes has passed into asparagine. Therefore it seems just to conclude that in the normal mature leaf there is a continuous production of asparagine from the reserve proteins.

Repeated researches since the time of Boussingault have shown that the concentration of asparagine in the green parts of plants grown under normal conditions is extremely low. Only under abnormal conditions, which produce a deficiency of carbohydrate, such as etiolating the plants, does any accumulation of asparagine occur [see Pfeffer, 1897, p. 462; Butkewitsch, 1908]. Since this is so it would appear that the asparagine formed by the protein degradation must be continuously translocated away from the leaf, and used by other parts of the plant for the protein synthesis necessary for growth and maintenance of life.

This fact is of great interest when compared with our already existing knowledge of the nitrogenous metabolism in germinating seeds, and in the formation of pods and seeds. In a series of papers Schulze and his co-workers at Zürich have shown that the metabolism of the reserve proteins in the seeds leads to a great increase of asparagine (or in certain exceptional cases glutamine), which is then translocated away to the growing parts and, as they believed, resynthesised to protein and other complex nitrogenous substances necessary for the newly formed cells. Similarly, during the ripening of seeds asparagine is translocated to the pericarp, and is then used for synthesis of protein in the seeds [Schulze and Winterstein, 1910, 2; Schulze, 1911].

All this evidence indicates that the rôle of asparagine in the metabolism of the mature plant is that of a translocatory substance, and that it is the chief medium whereby nitrogen in a form suitable for subsequent metabolic processes can be transferred from one part of the plant to another. Pfeffer considered that this was its chief function in the metabolism of seedlings. There is ample evidence, however, that asparagine can play another important part in plant metabolism. As Prianischnikoff [1916] has already shown with young plants, should conditions, such as derangement of the normal protein metabolism in the plant, give rise to ammonia, this substance, which is toxic in excess, is removed as asparagine. Table IV shows that the same reaction can occur under abnormal conditions in the mature leaf. As Prianischnikoff points out, in this respect asparagine in plant metabolism corresponds to urea in animal metabolism. The analogy, however, is misleading since urea cannot be utilised again by the animal body but is excreted, whereas asparagine remains stored up in the plant, and, under favourable conditions, can be subsequently utilised.

SUMMARY.

The decomposition products arising from protein metabolism in bean leaves have been investigated. These consist in large part of asparagine and other substances of undetermined composition containing free amino nitrogen.

The rôle of asparagine in the metabolism of the mature plant is discussed. It appears to be the chief agent whereby nitrogen in a form suitable for resynthesis of protein is conveyed from one part of the plant to another.

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LIII. INVESTIGATIONS ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS. PART VII.

LEAF PROTEIN METABOLISM IN NORMAL AND ABNORMAL RUNNER-BEAN PLANTS.

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The experiments described in the earlier papers of this series extend over the seasons 1921, 1922 and 1923. During this period sufficient data have been collected to demonstrate certain abnormalities, which, though they cannot yet be explained, appear to be worthy of note.

These relate chiefly to experiments made during the season 1921. That year a prolonged drought was experienced during July, August and September. The roots of the plants were watered daily, but in spite of a normal inflorescence during July very few pods formed. During August and September no new pods were formed at all, the plant activity being confined solely to the production of an abnormally large number of new shoots and leaves. The seasons 1922 and 1923 were normal, and pod production was continuous during August and September.

An interesting difference between the leaf protein metabolism in the normal and abnormal plants has been noted. This relates to the leaf protein degradation products, which accumulate in leaves detached from the plant and left with their petioles in water for some days. The chemical composition of these products in four separate cases, two of them normal and two abnormal, is illustrated by Table I. In this table "amide N other than asparagine N" means the ammonia set free after prolonged hydrolysis, from which the free ammonia N and amide N (Sachsse) have been deducted.

Case 1. Leaves from normal plants, picked early in September, 1922, during the pod forming stage. The protein N decreased to the extent of 14·85 % of the total leaf N, with a corresponding increase in the non-protein N. About one-half of this increase was due to an increase in asparagine N. "Amide N other than asparagine N" showed a small increase, whilst free ammonia N remained unchanged. This result was typical of several similar experiments quoted in an earlier paper [Chibnall, 1924].

Table I. *Showing the increases in various forms of non-protein N due to protein metabolism in the leaves of normal and abnormal plants.*

Phaseolus vulgaris *var.* multiflorus.

(In percentages of total leaf N.)

Case no.	Date picked	No. of days with petiole in water	I Total non-protein N	II Ammonia N	III Amide N (Sachsse)	IV Amide N other than asparagine N	Total of III and IV
1	11 Sept. 1922	0	16·75	0·46	1·06	1·44	2·50
	7 „	4	31·60	0·45	4·08	2·05	6·13
		Increases	14·85	—	3·02	0·61	3·63
2	12 July, 1923	0	14·05	0·30	0·75	n.d.	—
	12 „	4	37·74	1·01	5·63	„	—
		Increases	23·69	0·71	4·88	—	—
3	7 Aug. 1921	0	22·45	0·44	0·53	0·73	1·26
	23 „	4	34·14	0·44	0·49	5·69	6·18
		Increases	11·69	—	−0·04	4·96	4·92
4	11 Sept. 1922	0	16·75	0·46	1·06	1·44	2·50
	13 „	4	28·80	0·49	2·29	3·66	6·95
		Increases	12·05	—	1·23	2·22	3·45

Case 2. Leaves from normal plants, picked early in July, 1923, at the stage when flower-buds were about to form. As in Case 1, asparagine was the most prominent product of the protein metabolism. Free ammonia N showed a small increase: "amide N other than asparagine N" was not determined.

Case 3. Leaves from abnormal plants, picked 23rd August, 1921, during the drought. A more detailed description is given in an earlier paper [Chibnall, 1922]. The protein metabolism produced no increase in asparagine N, or in free ammonia N, but there was a large increase in "amide N other than asparagine N" equivalent to nearly one-half of the increase in total non-protein N. Such a result was totally different from that given by the normal leaves, for the products of the protein metabolism were clearly not simple amides such as asparagine or glutamine. This large production of ammonia on prolonged hydrolysis is very striking, and precludes the possibility of its being derived from soluble proteins or polypeptides, for these, like asparagine, yield practically all their ammonia during mild hydrolysis. It was at first thought that these unknown products might consist in part of urea or its derivatives, but a test for urea in similar material described in Case 4 showed it to be present only in traces. Lack of material has so far prevented any further study of this interesting observation being made.

Case 4. Leaves picked 13th September, 1922, from six abnormal plants. The seedlings had appeared above ground early in June, but, for reasons unknown, growth was stunted, and no flowers had formed. The protein metabolism produced a small increase in asparagine, but only about one-third of that given by the normal leaves. "Amide N other than asparagine N" showed a much larger increase than that given by the normal leaves, indicating the production

of the unknown products that were so pronounced in the protein metabolism of the abnormal leaves described in Case 3.

It seems probable, then, that under certain conditions, not yet known, the metabolism of the leaf proteins may vary, and it is interesting to note that in both cases when asparagine was not formed there was no production of pods.

In conclusion the author would like to thank Professors S. B. Schryver, V. H. Blackman and T. B. Osborne for their continued interest in the work described in this and the two preceding papers.

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LIV. OBSERVATIONS ON THE DISTRIBUTION AND VARIATION OF CARNOSINE IN CAT MUSCLE.

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(*Received February 25th, 1924.*)

In a previous paper [1922] it was shown that the carnosine content of muscle varies with the species of animal and with different members of the same species. The findings reported here deal only with cat muscle, and confirm previous observations. They further show that in the same animal carnosine is a variable constituent of white muscle, but is approximately constant in red.

Table I shows the carnosine content of several muscles of the right and left legs of six cats. The first value given in each case is for the right leg. The weights of cats Nos. 1, 2 and 5 were not recorded.

Table I.

Cat No.	Weight g.	Gastrocnemius % Carnosine	Soleus % Carnosine	Biceps femoralis % Carnosine	Semimembranosus % Carnosine	Semitendinosus % Carnosine	Vastus intermedialis % Carnosine
1	—	0·357	0·051	0·494	0·492	0·294	—
		0·350	0·053	0·483	0·490	0·278	—
2	—	0·251	0·067	—	—	—	—
		0·257	0·059	—	—	—	—
3	2680	0·038	—	—	—	—	—
		0·039	—	—	—	—	—
4	2780	0·037	—	—	—	—	—
		0·039	—	—	—	—	—
5	—	0·010	0·055	0·014	0·014	0·009	0·038
		0·010	0·062	0·013	0·011	0·009	0·032
6	1470	0·007	0·049	0·012	—	—	—
		0·007	0·048	0·011	—	—	—

It may be seen from the above table that (*a*) corresponding muscles in the same cat have the same carnosine content; (*b*) the carnosine content of the gastrocnemius, biceps femoralis, semimembranosus and semitendinosus is subject to wide variation in the different cats; (*c*) the carnosine content of the soleus muscle is constantly low and subject to smaller variation; (*d*) red muscle may have a lower *or higher* carnosine content than white muscle.

It may further be seen that the weight of the cat has no relation to the carnosine content of its muscle. Although not weighed, cats Nos. 1 and 2 were certainly smaller animals than 3 and 4, as could be judged from the weights of the whole muscles which were taken for each estimation. The significant difference between these two pairs of cats was that Nos. 1 and 2 were killed shortly after arrival at the laboratory, whereas Nos. 3 and 4 were

kept in cages for ten days. During this period they each consumed 60 g. of raw meat per day.

Cat No. 5 was very lean, having been partially starved during the week previous to killing. Cat No. 6 was starved for ten days in a cage, and was very weak before killing. That inanition does not wholly account for the low values obtained in cats Nos. 5 and 6 is indicated by the fact that the values obtained in cats Nos. 3 and 4 were likewise very low, although the cats were in excellent condition.

It was suspected that confinement might affect the carnosine; at any rate it was desirable to determine whether it was a fluctuating or constant constituent of muscle.

The procedure adopted was to amputate a leg, under chloroform, estimate the carnosine in various muscles, kill after several days and estimate the carnosine in the corresponding muscles of the other leg.

That the carnosine content did not fall during the actual period of anaesthesia was shown in the following way. A cat was anaesthetised with ether, and the right leg quickly severed near the middle of the femur. Chloroform was then substituted as anaesthetic and the animal kept under for 75 minutes. At the end of this time the animal was killed with the anaesthetic. The gastrocnemius and soleus were then tested in the corresponding legs, with results as follows:

Gastrocnemius before chloroform	0·251 % carnosine
Gastrocnemius after chloroform	0·257 ,,
Soleus before chloroform	0·067 ,,
Soleus after chloroform	0·059 ,,

Four cats were treated as described above, and the results are given in Table II. The first date in each case is the day on which the right leg was amputated; the second, the day on which the animal was killed and the muscles of the left leg estimated. The weights of the individual muscles are given in order that the degree of wastage may be noted.

Table II.

			Gastrocnemius		Plantaris		Soleus	
Cat No.	Wt. before operation g.	Date	Wt. of muscle g.	% Carnosine	Wt. of muscle g.	% Carnosine	Wt. of muscle g.	% Carnosine
7	2480	28. xi. 23	14·539	0·185	4·453	0·161	2·423	0·085
		7. xii. 23	11·660	0·054	3·703	0·048	2·138	0·090
8	2470	11. xii. 23	14·848	0·363	4·794	0·349	2·354	0·054
		22. xii. 23	11·159	0·020	3·236	0·015	1·945	0·058
9	2740	14. xii. 23	22·930	0·433	6·502	0·350	2·393	0·058
		24. xii. 23	16·839	0·020	4·719	0·015	2·083	0·059
10	2190	24. xii. 23	13·549	0·320	4·294	0·244	1·894	0·054
		28. xii. 23	11·879	0·164	3·534	0·094	1·820	0·053

Cats Nos. 8 and 9 were in the cage only a few hours before operation; cat No. 7 for three days, and cat No. 10 for ten days.

In all cases a good recovery was made after the operation. Cats Nos. 7 and 10 ate raw meat on the second day; cats Nos. 8 and 9 were slower in regaining their appetite.

Table II shows that (*a*) in the space of ten days the gastrocnemius and plantaris in cats Nos. 7, 8 and 9 dropped from one-quarter to one-twentieth of their original values, and that in the space of four days the values in cat No. 10 dropped to less than half; (*b*) the carnosine content of the soleus did not vary in the same cat, and varied only slightly in the different cats.

That carnosine varies in different muscles of the cat has recently been shown by Mitsuda [1923], who has also confirmed the writer's original observation that carnosine varies in the muscle of different animals of the same species. In the light of the results now reported this is to be expected. As has been shown, carnosine varies within wide limits in the same muscle of the same animal. As regards the lower limit, it is probable that it never entirely disappears. Even in cat No. 6, which was on the point of dying from starvation before it was killed, the gastrocnemius had 0·007 % carnosine[1].

One cannot be certain about the upper limit, but it seems highly improbable that even the semimembranosus reaches the value of 1·799 % carnosine as given by Mitsuda[2]. The highest value the writer has found in a gastrocnemius was in cat No. 9, *i.e.* 0·433 %. It is probable that the semimembranosus was between 0·5 % and 0·6 % in this case.

It is seen that there is a fairly constant relationship among the muscles which vary in their carnosine content. The semimembranosus and biceps femoralis have approximately the same values. Both are higher than the gastrocnemius, which in turn is slightly higher than the plantaris. The semitendinosus is still lower than the plantaris, and it may be noted that this relationship holds whether the carnosine content be high or low.

The results are more suggestive than enlightening. They suggest that the carnosine content of muscle falls with confinement or want of exercise. Even when the cats were fed on butcher's meat the values continued to fall. The difficulty experienced by some in the preparation of carnosine may be in part explained by the fact that some butcher's meats have a low carnosine content. The writer has found ox muscle with less than 0·1 % carnosine. It is possible

[1] This is not a very reliable figure, because the azo colour developed in such conditions is very yellow; yet it is remarkably proportional, and has about the same developmental time as pure carnosine solutions. The relatively greater amount of potentially interfering substances may easily account for the yellow colour [see Hunter, 1922].

[2] There is either an error or a misprint in Mitsuda's paper. He makes a standard carnosine solution containing 0·00001 g. of carnosine nitrate in 1 cc., of which he states 0·3 cc. contains 0·03 mg.! Does this mean that his values are only one-tenth of those published?—in which case they would represent cats with rather low but more probable values. That the discrepancies between our findings are due to an error in standards or calculations is more probable than that they are due to actual differences in different animals. The almost constant values obtained for the soleus of different animals point to this conclusion.

It would seem desirable that future workers on carnosine should give the molecular colour value—as measured against Koessler and Hanke's [1919] colour standard for histidine—of the carnosine used as standard. If this were done there would then be a definite standard of comparison among the results of different workers, and one could perhaps trace the source of discrepancies. While in Prof. Cathcart's laboratory in Glasgow, the writer published his molecular colour value as measured from carnosine, and from carnosine nitrate. He has again prepared carnosine since coming to Canada, has reconfirmed his previous results and further reinforced these by measurement of the molecular colour value from carnosine-copper. [Clifford, 1921; Mitsuda, 1923; Hunter, 1921, 1922.]

that the muscle of stall-fed cattle has a lower carnosine content than that of animals grazed in the open.

Why there should be a fairly constant relationship in the carnosine content of those muscles with fluctuating values, or why the soleus content should be low but constant, is quite obscure. It would be premature to say that carnosine is unique in this manner of variation, as very little attention appears to have been given by physiologists to the chemistry of individual muscles. Consideration of the function, blood or nerve supply of the various muscles offers no obvious explanation.

Mere mention may be made here of the behaviour of the urines towards the diazo reagent in these cases. One might suspect that some carnosine or derivative of that substance would appear in the urine when carnosine rapidly disappears from muscle. Indications of this have indeed been obtained. A further report on this subject is pending.

SUMMARY.

1. Corresponding muscles in the cat have the same carnosine content.

2. The carnosine content of the same white muscles in different cats is subject to wide variation. This confirms Mitsuda's finding, although the absolute values obtained by Mitsuda are questioned. In the gastrocnemius, the highest and lowest values found were 0·433 % and 0·007 % carnosine.

3. The carnosine content of red muscle, *e.g.* soleus, of different cats is always low, and subject only to slight variation. The highest and lowest values found were 0·090 % and 0·048 % carnosine.

4. The carnosine content of white muscle in the same cat fluctuates. Lack of exercise is suggested as a possible cause.

5. The carnosine content of red muscle in the same cat, *e.g.* soleus, does not show this fluctuation.

6. There is a fairly constant relationship between the carnosine contents of the fluctuating muscles, whether the values be high or low.

7. The same muscles in different cats show as wide a variation as different muscles in the same cat. The carnosine content of red muscle is not necessarily lower than that of white muscle. In these two points the writer differs from Mitsuda.

The writer is indebted to Prof. V. J. Harding for suggestions; to the Medical Research Committee, University of Toronto, for the animals used; and to Mr W. Cowan for his care of the animals and help received throughout the work.

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LV. NOTE RELATIVE À LA DÉFICIENCE EN VITAMINES *A* CHEZ LE PIGEON.

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(*Received February 26th, 1924.*)

NOUS avons, dans un article paru dans cette revue même [1923], donné nos premiers résultats obtenus sur pigeons avec une alimentation artificielle déficiente en vitamines A. Nous nous étions décidé à les publier, malgré la durée de nos expériences, jugée par nous-même insuffisante, parce qu'un travail presque identique de Benedict et Sugiura [1923] avait conduit ces auteurs à nier la nécessité des vitamines A pour les pigeons, jeunes et adultes. Et de fait, après cinq mois de régime artificiel déficient nous ne constations nous-même de manifestation anormale chez aucun de nos animaux. Nous pensions donc, à ce moment-là, sous la réserve exprimée quant à la durée de nos recherches, pouvoir confirmer les conclusions de Benedict et Sugiura. Les faits que nous avons observés dans les mois qui ont suivi et que nous relatons brièvement, nous forcent à revenir sur ces conclusions.

Au moment où nous rédigions nos premières observations, aucun de nos six pigeons, trois mâles et trois femelles (nº 26, 27, 50, 51, 52 et 53), maintenus depuis cinq mois à un régime artificiel uniquement déficient en vitamines A, ne présentait de symptômes morbides. Le graphique II de notre précédent mémoire montre les courbes pondérales de ces animaux. Le pigeon nº 51 avait eu deux œufs couvés normalement; les jeunes pigeons éclos à terme étaient toutefois morts après trois et cinq jours; le gésier de l'un était vide, celui de l'autre contenait dix grammes de nourriture. Peu de temps après, la femelle nº 26 à son tour pondait et couvait deux œufs, l'un, après trois semaines d'incubation, donnait naissance à un petit qui mourrait au deuxième jour, tandis que l'autre contenait un embryon mort. Observation absolument identique chez la troisième femelle nº 52, au cours du septième mois. Après ces deux uniques œufs chez chacune de nos trois femelles, la ponte s'est arrêtée comme si les vitamines A en réserve dans l'organisme, et nécessaires à la formation des jaunes, avaient été épuisées.

A partir du sixième mois, tous nos pigeons ont cessé d'être normaux bien que d'une façon très inégale. Progressivement est apparue chez cinq d'entre eux, une faiblesse des pattes et des ailes comparables au "leg weakness" dont parlent les éleveurs et expérimentateurs américains. Leur démarche devient particulière, difficile, les pattes sont traînantes; très souvent on les trouve

couchés sur le dos, dans l'impossibilité de se relever, ou immobiles, reposant sur la poitrine, la tête en avant, les pattes étendues en arrière. Le pigeon n° 52, plus gravement atteint, meurt huit mois après le début du régime à l'huile d'arachides, ayant perdu une cinquantaine de grammes. L'état des autres reste précaire, leur poids cependant ne varie guère; chose étonnante, l'addition de beurre au régime vers le neuvième mois n'exerce aucune influence.

Nous nous décidâmes alors d'interrompre l'alimentation artificielle et de remettre les animaux au maïs ordinaire. En dix à douze jours, tous avaient repris une allure normale, et moins d'un mois après, la ponte avait repris chez l'un d'entre eux, le n° 51. Ajoutons que différents essais d'alimentation de jeunes pigeons normaux avec une nourriture déficiente en A ont tous échoués.

Nos expériences ne nous permettent donc pas de confirmer les résultats de Benedict et Sugiura. Il ne semble pas possible, pour peu que l'on prolonge les essais, de supprimer impunément les vitamines A de l'alimentation des pigeons.

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LVI. EXPERIMENTAL RICKETS IN RATS ON A PURIFIED SYNTHETIC DIET DEFICIENT IN PHOSPHORUS AND FAT-SOLUBLE ORGANIC FACTOR[1].

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(*Received March 3rd, 1924.*)

Introduction.

Sherman and Pappenheimer [1921] and later, Pappenheimer, McCann, Zucker and Hess [1921] induced the development of severe experimental rickets in rats by a diet (No. 84) consisting of 95 % patent flour and 5 % salt mixture. This diet was not only deficient in phosphorus and fat-soluble A, but was also very obviously deficient in many other respects. McCollum, Simmonds, Shipley and Park [1921], by means of diets (No. 3143 and No. 3127) composed of complex natural food substances deficient in phosphorus and the fat-soluble organic factor, also succeeded in inducing the development of severe rickets in rats. Later, McCollum, Simmonds, Becker and Shipley [1922], using diets (No. 3407 and No. 3408) composed essentially of purified food substances and deficient only in phosphorus and the fat-soluble organic factor, again succeeded in producing severe rickets in rats. On the other hand, Korenchevsky [1921] failed to produce rickets in rats by feeding them on a purified synthetic diet, referred to as − A-P diet, deficient in phosphorus and the fat-soluble organic factor. He suggests that the discrepancy between his results and that of the other authors "is no doubt due to the difference in phosphorus content in the basal diets used." But the following table shows that his − A-P diet had a lower percentage of phosphorus than some of the diets of McCollum and his co-workers. In a later communication, Korenchevsky [1922] recognised this but laid emphasis on the fact that the composition of the diet in his experiments was entirely different from those of McCollum and his co-workers.

[1] In this communication the term "fat-soluble organic factor" is used to denote the deficiency of the diet in both the fat-soluble growth-promoting and anti-rachitic organic factors. During the process of purification of the caseinogen by the heat and oxidation method, presumably both factors were completely destroyed.

Table I.

	Diet	Phosphorus %	Calcium %	Weight ratio calcium : phosphorus
Sherman and Pappenheimer	84	0·087	0·553	1 : 0·15
McCollum, Simmonds, Shipley and Park	3143	0·301	1·231	1 : 0·25
McCollum, Simmonds, Becker and Shipley	3407	0·217	1·202	1 : 0·18
Do. Do.	3408	0·088	1·202	1 : 0·07
Korenchevsky	− A-P	0·22	0·256	1 : 0·86

Table I shows that in all but the − A-P diet there is a very definite disproportion between the calcium and phosphorus contents, the former being relatively high. Now McCollum and his co-workers [1921] have drawn particular attention to this disproportion in the calcium-phosphorus ratio as an important factor in making their diets severe rickets-producing ones, and have suggested that the "physiological relation in the diet between the calcium and phosphate is of infinitely greater importance in insuring normal calcification than the absolute amount of the salts themselves." Sherman and Pappenheimer [1921] also state that "the quantitative relations of the inorganic ions rather than an absolute deficiency of any one of them may have been the determining factor" in the causation of the rickets which was induced by their diets Nos. 83 and 84.

It seemed probable that the absence of this disproportion between calcium and phosphorus in the − A-P diet of Korenchevsky might account for his failure to produce rickets with it. Korenchevsky, in his monograph on Rickets [1922], admitted the possibility of such an explanation. The following experiments were therefore performed in order to elucidate this point.

Experimental.

Each of two litters of rats was divided into three groups. One group was put on the following diet which is similar[1] in almost every respect to the − A-P diet of Korenchevsky, and will also be referred to as − A-P.

− A-P diet

Caseinogen (purified)	20 g.
Starch	50 „
Cotton-seed oil (hardened)	15 „
Salt mixture (No. 4 [Korenchevsky, 1922])	5 „
Marmite	5 „
Lemon juice	5 cc.
Distilled water	50 „

Another group was put on the same diet, to which, however, was added sufficient calcium carbonate to raise the calcium-phosphorus ratio from 1 : 0·88 to 1 : 0·20. This will be referred to as the "− A-P + Ca" diet.

A third group was put on a control diet which was the same in all respects as the − A-P diet described above, with the exception that the salt mixture contained sufficient phosphorus to make good the deficiency. In order to supply the necessary amount of the fat-soluble organic factor, five drops of

[1] Korenchevsky's − A-P diet contained only 18 % caseinogen and orange, instead of lemon, juice.

a very potent cod-liver oil were administered daily to the rats. One-fifth of a drop of this oil given daily was sufficient to promote growth in rats on a fat-soluble A deficient diet and to protect rats on McCollum's diet No. 3143 from developing rickets. The percentage of calcium and of phosphorus in these three diets and their ratios by weight are given in Table II.

Table II.

Diet	Calcium %	Phosphorus %	Ratio by weight calcium : phosphorus
Normal	0·25	0·54	1 : 2·16
− A-P	0·25	0·22	1 : 0·88
− A-P + Ca	1·08	0·22	1 : 0·20

The detailed results given in Table III show that in each litter the rats on the normal diet were normal in every respect. The bones of the rats on the − A-P diet showed only a slight degree of osteoporosis histologically, and a deficiency of 12 % in the calcium. This agrees with the results obtained with the same diet by Korenchevsky. But all the rats on the − A-P + Ca diet developed rickets of from moderate to very severe degree. This was evident even grossly and was verified in every case by histological examination of the ribs. In these rats, chemical examination of the bones (femur, tibia and fibula of both legs) showed in every case a very great deficiency in calcium as compared with those on the normal and − A-P diets. As is usual in rickets, the percentage of water in the bones of the rats on diet − A-P + Ca was greatly increased (see Table III).

As part of another investigation, 16 rats from three litters were put on the − A-P + Ca diet. In every rat rickets developed which varied in degree from moderate to very severe. The average percentage of calcium in their bones was 6·36 % per dry and 14·38 % per wet weight of the bones. This is mentioned here merely to show that the − A-P + Ca diet can be depended upon to produce some degree of rickets in practically every young rat fed on it for from six to eight weeks.

Another group of 14 young rats from four litters were put on the − A-P + Ca diet, but with the caseinogen replaced by cooked commercial egg-white. Egg-white contains less phosphorus than caseinogen, so that this diet had only 0·08 g. of phosphorus per 100 g. of the wet paste. On this diet the rats all developed severe rickets in from four to five weeks, more rapidly therefore than on the − A-P + Ca diet which contained caseinogen. The average percentage of calcium in their bones was also lower, being 4·22 % per wet and 10·30 % per dry weight of the bones. But the two groups are not strictly comparable, since they were not from the same litters. Whether the greater rickets-producing power of the egg-albumin diet was due to the lower percentage of phosphorus or to the different protein, or to both, cannot be stated.

Table III.

No. of rat	No. of litter	Sex	Diet	Initial age days	Final age days	Days on diet	Initial weight g.	Final weight g.	Gain in weight g.	Av. amount of food eaten daily g.	In bones: % H_2O	In bones: % Ca wet	In bones: % Ca dry	Histological diagnosis
G 906	XXVII	♀	Normal	37	99	62	33	104	71	12·6	41·65	13·13	22·28	Normal
G 907	,,	♀	,,	37	99	62	38	111	73	12·6	39·98	12·51	21·52	,,
G 913	XXVIII	♂	,,	26	77	51	32	179	147	14·7	41·17	12·46	21·17	,,
G 914	,,	♂	,,	26	77	51	32	113	81	14·7	40·57	12·08	20·36	,,
										Averages	40·84	12·54	21·53	
G 908	XXVII	♀	−A-P	37	99	62	32	99	67	12·4	43·67	12·12	21·52	Nearly normal
G 909	,,	♀	,,	37	99	62	31	115	84	12·4	41·06	11·85	20·15	Osteoporosis slight
G 915	XXVIII	♂	,,	26	77	51	34	99	65	—	39·75	11·39	18·89	Nearly normal
G 916	,,	♂	,,	26	77	51	36	103	67	—	45·99	9·35	17·32	Osteoporosis moderate
G 917	,,	♂	,,	26	77	51	36	88	52	—	41·29	10·39	17·69	,, slight
										Averages	42·35	10·92	19·40	
G 910	XXVII	♀	−A-P+Ca	37	99	62	28	68	40	8·9	52·35	7·15	15·06	Rickets slight (healing)
G 911	,,	♀	,,	37	99	62	27	62	35	8·9	53·00	7·84	16·71	,, moderate
G 912	,,	♀	,,	37	99	62	30	94	64	8·9	52·82	7·67	16·28	,, severe
G 918	XXVIII	♂	,,	26	77	51	40	62	22	10·3	57·86	6·26	14·86	,, moderate
G 919	,,	♂	,,	26	77	51	37	74	37	10·3	58·33	5·30	12·74	,, severe
G 920	,,	♂	,,	26	77	51	38	79	41	10·3	53·77	6·62	14·35	,, ,,
										Averages	54·68	6·80	14·99	

Discussion.

From the results shown in Table III it is quite evident that the mere addition of calcium carbonate to Korenchevsky's −A-P diet was sufficient to transform it from a non-rickets-producing into a very definite rickets-producing diet. The addition of the calcium carbonate raised the proportion of calcium to phosphorus in the diet from 1 : 0·80 to 1 : 0·20. Whether it is the increase in the calcium content, and, consequently, the greater disproportion between the two elements calcium and phosphorus, or merely the increased basicity which is responsible for this change in the character of the diet cannot be stated. The administration to some of the rats of the −A-P diet with sodium carbonate instead of calcium carbonate added to it might have helped to clear up this point. The investigations of McCollum, Simmonds, Shipley and Park [1922] led them to the belief that the reaction of their diets did not affect their rickets-producing power. They found that the substitution of calcium chloride, which contains a non-oxidisable acid radical, for the carbonate or lactate, which are both potential neutralisers of acid in the body, had no effect on the rickets-producing character of their diets. On the other hand, Zucker, Johnson and Barnett [1922] state that the acid-base ratio of their diet was of the utmost importance in determining its rickets-producing quality. The substitution of calcium chloride for the calcium lactate, or the addition of ammonium chloride to the diet, had the effect of changing it from a rickets-producing to a non-rickets-producing one, while the addition of sodium carbonate to their non-rickets-producing diet changed it to a severe rickets-producing one. In view of these divergent results the influence of the acid-base ratio on the rickets-producing quality of a diet must still remain an open question.

Conclusions.

To a purified synthetic diet low in phosphorus and the fat-soluble organic factor, the addition of calcium carbonate in such an amount that the proportion of calcium to phosphorus was raised from 1 : 0·88 to 1 : 0·20 had the effect of changing it from a non-rickets-producing to a rickets-producing one.

It is with sincere pleasure that I acknowledge my thanks to Prof. C. J. Martin for permission to carry out this and other investigations at the Lister Institute, as well as for his constant help and advice. I also desire most heartily to thank Miss K. M. Soames for much technical assistance in this investigation.

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LVII. AN INEXPENSIVE FURNACE FOR ASHING FOOD AND EXCRETA IN PORCELAIN CRUCIBLES.

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(*Received March 3rd, 1924.*)

In connection with metabolism experiments in my laboratory it has been required to incinerate numerous samples of about 100 g. of food and excreta with a view to the determination of calcium and phosphate in the ash.

Large platinum basins and an electric muffle being unavailable, the little furnace described below was improvised. It has proved satisfactory and may help others to get over similar difficulties.

The ashing takes place in a large porcelain crucible and the furnace is constructed of simple materials available to any laboratory.

The requisites for combustion in porcelain are

(i) uniform temperature well below that sufficient to fuse the ash or the glaze, or to vaporise any constituent of the ash which it is proposed to determine;

(ii) adequate air supply.

From the sketch, which is drawn to scale, the principle and construction of the furnace will be obvious.

The crucible is surrounded by a short length of so-called asbestos flue-pipe 5 ins. in diameter. This pipe is made of a mixture of asbestos and cement. It is a common article of commerce and procurable in various sizes. At one-third of its length, holes are bored to support three pieces of $\frac{3}{16}''$ iron rod, inserted so as to furnish a triangle on which the crucible rests.

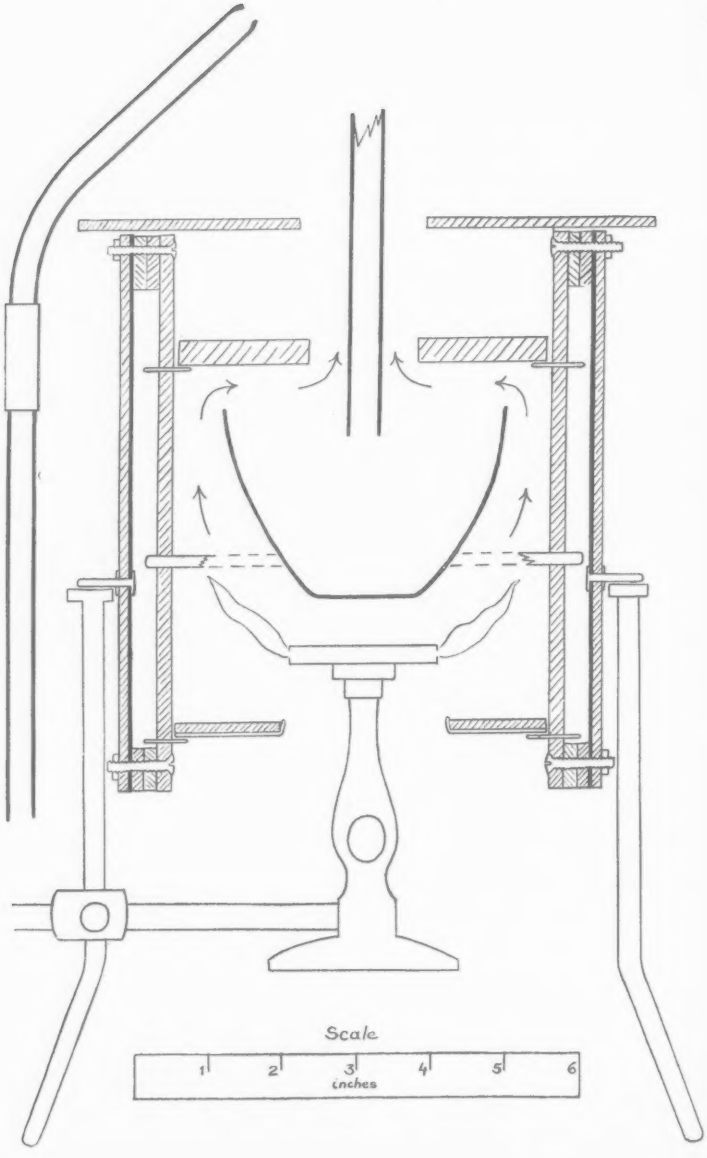
Below the crucible is a solid rose burner and below this a diaphragm of metal resting on three pins and supporting a ring of asbestos.

Half an inch above the crucible, also resting on three pins, is a disc of silica $\frac{1}{2}''$ thick with a central hole[1].

The hot gases thus completely enclose the crucible, and the roof, floor and sides of the furnace attain a uniform bright or dull red heat as required. The top of the rose burner also becomes red hot but the stem is kept cool by the draught of cold air.

To economise heat the furnace is surrounded by a thin metal cylinder (a large tin with the bottom removed) leaving about $\frac{1}{2}''$ air space between, and

[1] Made for me by the Thermal Syndicate, Walsend-on-Tyne, for a few shillings each.


Scale
1
2
3
4
5
6
inches

the tin is wrapped around with asbestos. This outer insulation is fixed to the furnace at the top and bottom by a ring of asbestos packing and secured by small stove-bolts and nuts.

On the top is placed a piece of "Poilite" with a central hole, for further insulation.

It is important to keep to approximately the proportions shown to obtain a satisfactory draught.

The furnace is supported on a tripod stand with circular ring by three projecting pins (copper rivets).

The arrangement satisfies the first requirement stated and with a surprisingly small gas flame, once the furnace is heated. It does not, however, supply oxygen, for the crucible is enveloped in the products of the combustion of the gas, diluted with air. To secure a supply of oxygen, a silica tube of about 1 cm. bore is inserted from above until within about 1 cm. of the ash and a steady current of air supplied from a Bunsen filter pump at the rate of 1–2 litres per minute. This gives a current at the mouth of the tube of 25–50 cm. per second. The rate of combustion is almost entirely dependent on the volume of air delivered. A brisker current than that mentioned above is permissible with most materials.

The silica tube is joined to a piece of glass tubing, bent to an elbow, by a metal tube with asbestos packing. It is held in position by a clamp and retort stand.

To adjust the air supply it is useful to bubble through water and control the rate by counting the bubbles. Oxygen can be used instead of air provided there is little carbon left, otherwise the action is too fierce and melts the mouth of the silica tube. The preliminary burning is best done without the silica roof to the furnace as it can be more easily controlled.

LVIII. AN OBSERVATION ON THE VALUE OF EGG-WHITE AS THE SOLE SOURCE OF NITROGEN FOR YOUNG GROWING RATS.

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(*Received March 3rd, 1924.*)

Osborne and Mendel [1913, 1, 2; 1915] state that egg-white gave adequate growth in rats, provided that a sufficient quantity was given, *e.g.* 18 % of the total diet.

Bond [1922] also obtained excellent growth in rats on a diet in which egg-white formed 20 % of the total diet, and was the sole source of protein. In my experiments egg-white formed 20 % of the diet. The egg-white employed was a dried commercial preparation of Chinese origin (the same as that used by Bond).

The composition of this diet was as follows:

Egg-white	41 g.	Hardened cotton-seed oil	30 g.
Wheaten starch	113 g.	Salt mixture	3·5 g.
Marmite	12 g.	Ferric citrate	0·14 g.
Orange juice	12 cc.	Distilled water	93 cc.

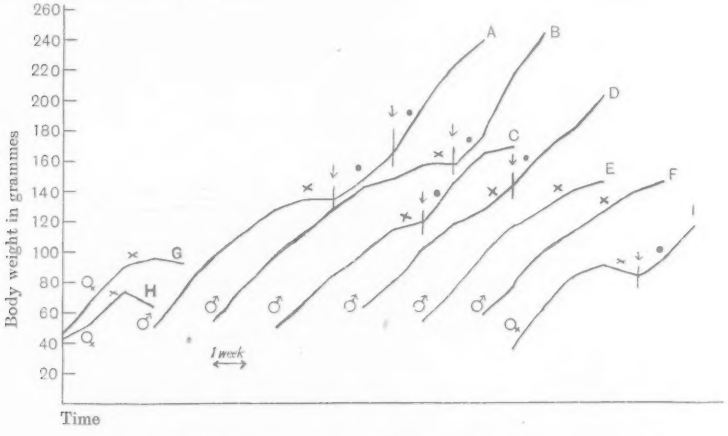
The egg-white was soaked over night in the water before adding it to the rest of the diet. For further details of this diet see the following paper [Boas, 1924]. Fat-soluble vitamins were supplied in the form of 3–5 drops of cod-liver oil per day per rat.

At first this diet appeared perfectly satisfactory. Subsequently it became clear that when rats reared on it were growing rapidly, 20 % egg-white in the ration was not adequate to supply the nitrogen requirements. The animals grew well, appeared in health for from four to six weeks, but after that time loss of hair occurred, and those parts where most rubbing took place, rapidly became bald. This was often accompanied by blepharitis. At the same time the weight remained stationary for a week or so, and then fell. The substitution of caseinogen or serum-albumin for the egg-white always cured the condition, new hair appearing in less than a week. The rats soon regained their lost weight. The deficiency could also be met by green food in the form of spinach given in a daily ration of 5 g. per rat, or by 5 cc. of fresh milk daily. Animals on the above diet without cod-liver oil showed the condition in a much less acute form. The substitution of

gluten or gelatin for half the egg-white seemed to give a certain amount of protection.

Wherein the deficiency of egg-white lies, has not been determined, but a paper by Harris [1923] which appeared while this work was being done gives a possible clue. In this very interesting paper the author showed that 86 % of the total sulphur in egg-albumin was not in the form of cystine, as had been supposed, but in an unidentified grouping. In serum-albumin on the other hand 89 % of the total sulphur was found to be in the form of cystine. As a prominent symptom, when egg-white was the sole source of protein, was cessation of growth and falling out of hair, and as the high content of cystine is the characteristic of keratin, the observations of Harris point to the possibility of a deficiency of cystine being the cause of the condition. Against this, however, is the fact that the trouble was cured by a diet of 20 % caseinogen as well as by serum-albumin. The amounts of cystine in proteins are notoriously under-estimated, but even so it is difficult to suppose that caseinogen would contain more than egg-white.

The growth curves of some of the rats in the above experiments are shown in the accompanying figures.



× Hair falling • Hair growing again.

Fig. 1. *Growth curves of rats on egg-white diet, supplemented with cod-liver oil.*

Curve A. Male rat on 20 % egg-white. At the first arrow the 20 % egg-white was changed to 20 % serum-albumin. At the second arrow the rat was put on to mixed diet (bread, fresh milk, oats, bran and cabbage).
Curve B. Male rat on 20 % egg-white. At the arrow, the rat was put on to mixed diet.
Curve C. Male rat on 10 % egg-white, and 10 % gluten flour. At the arrow the protein in the diet was changed to 20 % caseinogen diet.
Curve D. Male rat on 10 % egg-white and 10 % gelatin. At the arrow the protein was changed to 20 % caseinogen diet.
Curve E. Male rat on 10 % egg-white, and 10 % gelatin.
Curve F. Male rat on 10 % egg-white, and 10 % gluten flour.
Curve G. Average figures of two female rats on 20 % egg-white.
Curve H. Average figures of four female rats on 20 % egg-white.
Curve I. Female rat on 20 % egg-white. At the arrow, it was put on to mixed diet.

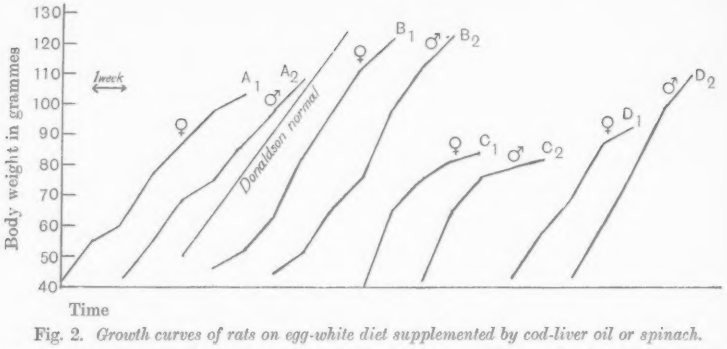


Fig. 2. *Growth curves of rats on egg-white diet supplemented by cod-liver oil or spinach.*

Curves A_1, A_2, B_1, B_2 show the growth of rats of the same litter. In A_1 and A_2 the basal diet was supplemented by five drops of cod-liver oil daily. (These rats had very bad coats.) In B_1 and B_2 the diet was supplemented by 5 g. of fresh spinach daily. (These rats had excellent coats.)

Curves C_1, C_2, D_1, D_2 show the growth of rats of the same litter. In C_1 and C_2 the diet was supplemented by three drops of cod-liver oil. (The rats had bad coats.) In D_1 and D_2 the rats received 5 g. of spinach daily in addition to the basal diet. (The rats had good coats.)

Summary.

Egg-white has been found inadequate as the sole source of nitrogen for young growing rats, even when it amounts to 20 % of the total ration.

My thanks are due to Prof. C. J Martin for his help and criticism while this work was being carried out.

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LIX. A METHOD FOR ESTIMATING THE RETENTION OF CALCIUM AND PHOSPHORUS IN YOUNG GROWING RATS.

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(*Received March 3rd, 1924.*)

The measure of calcium and phosphorus retention week by week during the period of active growth and the effect produced upon it by varying different factors is essential in the study of rickets and other disorders of calcium phosphate metabolism.

A diminished calcium retention will be demonstrable for some time before the summation of its effect will be sufficient to produce a lowered calcium content of the bones, outside the normal variation, and a determination of the calcium and phosphorus retention over a considerable period is therefore a more delicate and convincing test for abnormal calcium metabolism than any other available. A certain amount of work of this kind has been done on children by Birk [1910], Dibbelt [1910], Orgler [1911], Schabad [1910] and more recently by Findlay, Paton and Sharpe [1921] and Holt, Courtney, and Fales [1920]. Schabad [1910] pointed out that before any clinical signs of rickets occur the approach of the malady is indicated by a low positive, or even a negative, calcium balance.

The results of all these workers have been summed up by Korenchevsky [1922]. A lowered calcium retention was observed in most cases of rickets but as the period of observation was usually short, the general course of calcium metabolism is not apparent. Calcium retention has also been studied by Hart, Steenbock, and Hoppert [1921] on goats, by Meigs, Blatherwick and Cary [1919] and Hart, Steenbock, Hoppert and Humphrey [1922] on milking cows, but as these observations were carried out on adult animals no information as regards the metabolism during growth can be obtained from them.

In view of the large extent to which the rat has been used in the experimental investigation of rickets, it was thought that a series of studies of calcium and phosphorus metabolism in this animal upon various diets and over a considerable period of its growth would be of value. McClendon [1922] has estimated the calcium and phosphorus retention of a number of rats on

normal and rickets-producing diets, but as a rule his observations were carried on for one week only. He found in normal animals of about 30 g., a daily retention of calcium varying from 10 to 20 mg. and of phosphorus from 6 to 10 mg., whereas in the animals on deficient diets, both balances were much reduced. In one experiment on normal animals he observed the calcium and phosphorus balances over a period of one week when the animals were 28 days old and also during a period of three days when they were 57 days old. During the second period he found a daily retention which was double that found in the earlier period. At the same time it must be noted that the rate of growth was also much increased.

To arrive at a trustworthy balance sheet showing the weekly income and expenditure of calcium and phosphorus necessarily involves much labour and careful manipulation. The method described below has been evolved after a considerable amount of preliminary work. It has been repeatedly tested on rats receiving both normal and deficient diets and has proved satisfactory.

Experimental Method.

The diet used had the following composition:

Egg-albumin	41 g.	"Hardened" cotton-seed oil	5 g.
Wheaten starch	113 g.	Salt mixture	3·5 g.
Cod-liver oil	12 g.	Ferric citrate	0·14 g.
Marmite	12 g.	Distilled water	93 cc.
Orange juice	12 g.		

The salt mixture had the following composition:

Sodium chloride	13 g.
Sodium bicarbonate	53 g.
Magnesium carbonate	14 g.

The egg-white was soaked in the water over night. The starch and salts were mixed together and to them the cod-liver oil and marmite were added. The egg-white was cooked and stirred into the mixture; the hardened cotton-seed oil was melted and added, and last of all the orange juice was poured in. The whole was then mixed with extreme thoroughness. The diet was made once a week and kept in a refrigerator room.

Analyses of the freshly made diet gave a Ca content of 0·0261 % and a P content of 0·1402 %.

The experiments were usually started as soon as the rats were weaned, *i.e.* when they were between three and four weeks old and weighed 40 to 50 g. At first four rats of the same sex and litter were kept in one cage but it was afterwards decided to limit the number to two. Each experiment lasted five or six weeks and was divided into periods of one week each. The first week was used as a preliminary period during which no analyses were made, to enable the rats to become acclimatised to the basal diet and to the metabolism cages.

The cages used were those designed by Ackroyd and Hopkins [1916] but a few modifications were introduced, the most important being a detachable wire floor, which greatly facilitated the cleaning of the cage. It was found that the glass separators used by Ackroyd and Hopkins did not ensure quantitative separation of urine and faeces but gave a rough separation which greatly assisted the subsequent analysis. When more than one rat was kept in one cage, it was found impossible to prevent food being brought back into the cage, and so the inner tunnels for the side arms were not used, as they served no useful purpose and only rendered the work of cleaning more complicated. Food was given in glass dishes in one or both of the side arms, and distilled water was placed in a bulb fixed in the roof of the cage.

Determination of Intake. Sufficient diet to last for six days was weighed out to the nearest g. in a wide-mouthed stoppered bottle. To this was then added sufficient calcium carbonate and potassium phosphate (K_2HPO_4) to give 0·7 to 0·9 % Ca and 0·6 to 0·8 % P in the final mixture. These were well mixed in, so that a diet of uniform constitution was obtained. The amounts of salts added were accurately weighed to the third place of decimals. The $CaCO_3$ was found on analysis to contain 40·0 % Ca. The K_2HPO_4 was heated at 110° for three hours before weighing; it contained 17·64 % P. Both carbonate and phosphate were kept in bottles with paraffined stoppers. On the seventh day of each week the basal diet only was fed to the rats, without addition of Ca and P salts. This was done in order to prevent as far as possible Ca given in one week being excreted during the subsequent one. Thus the total weight of Ca and P given in each week to each cage could be calculated from the known weights of fresh diet and of added $CaCO_3$ and K_2HPO_4. From this amount was subtracted the total amount of Ca and P recovered from the cage in the course of the week (*i.e.* in excreta and any uneaten food). The remainder was therefore the amount retained in the bodies of the rats. It was most important that the method of recovery should be as efficient as possible, since all error would fall upon this difference, that is upon the retention which was being determined.

Estimation of Output. Although each metabolism period consisted of seven days, collection of excreta, etc. was made every second day. While this was being done the cage was removed from the funnel and placed over a large porcelain photographic dish, which could be afterwards rinsed into a large evaporating dish used to collect the urine. The solid matter consisting of faeces and uneaten food was put into a porcelain crucible. After each collection the crucible was put into a drying oven, and the contents when dry were partly ashed before the next collection of excreta.

In later experiments the amount of uneaten food was approximately determined. It was removed from the food-pots, any particles that had fallen into the faeces being separated by hand, and dried in a small porcelain crucible to constant weight, before adding it to the rest of the material to be ashed. When the seven day period was up, the weekly cleaning of the cage

took place. After the solid matter and urine had been removed as usual, the cage and glass parts were cleaned with scrupulous care with distilled water, a glass rod with a rubber tip and pieces of ash-free filter paper being used to ensure the removal of all adherent particles. These washings together with those from the diet bottle and the porcelain dish (on which the rats had been placed under a zinc cover during the cleaning) were added to the urine in the evaporating dish and the whole dried over a water-bath. When they were reduced nearly to dryness the contents of the evaporating dish were added to the partly ashed solids in the crucible and the whole dried in the oven at 105°–110°. As the dry material often amounted to as much as 100 g. in weight the ashing of it presented a problem of considerable difficulty. The large size needed put platinum crucibles out of the question, nickel was found to be attacked by some of the constituents of the ash, and silica was also unsatisfactory owing to the formation of calcium silicate. Porcelain crucibles were selected and proved satisfactory. They were used in the special furnaces designed by Prof. Martin [1924]. When the crucible was first placed in the furnace the silica and poilite plates were removed and only a very small flame was used. The material was allowed to ash slowly in this manner until the smoky stage was finished. Then the plates were replaced, the flame turned up gradually and a slow stream of air admitted. When a fairly white ash had been obtained, usually about three hours after the completion of the first stage, the crucible was allowed to cool slowly. When cool the ash was moistened with distilled water, strong HCl was added drop by drop until effervescence ceased and then 3–4 cc. in addition. The whole was allowed to stand for a few hours or over night, after which the supernatant fluid was pipetted off and filtered through ash-free filter paper into a calibrated litre flask. One more extraction with strong HCl was followed by repeated extractions with small quantities of distilled water. The well-washed filter paper and its contents were then replaced in the crucible, dried, re-ashed and extracted as before. This double ashing usually produced a completely soluble ash, but any insoluble matter still remaining was collected on a second filter paper, re-ashed and treated for a long time with strong HCl and water and then tested for the presence of calcium. Not more than the faintest trace was ever found.

The solution in the flask was made up to 1 litre and aliquot portions taken for calcium analysis, the quantities being measured in a calibrated flask. The method of analysis adopted was based on those of McCrudden [1909, 1911] and Shohl [1922]. The final determination was, however, gravimetric. It was as follows. To the measured volume of solution which was strongly acid with HCl, 10 cc. of 10 % oxalic acid solution and three drops of methyl red were added, and afterwards strong ammonia drop by drop until the solution was alkaline; the solution was then made definitely acid again with 2 *N* HCl and heated on a water-bath until the precipitate was granular. When cool, 20 % sodium acetate solution was added until a p_H of 5·0 was reached. (The danger limits are 4·0 and 5·6.) After standing over night the

precipitate was filtered through ash-free filter paper and washed with 0·5 % ammonium oxalate solution. The paper and contents were then transferred to a weighed platinum crucible and converted to a white ash. When quite cool about 0·3 cc. of strong H_2SO_4 was added very cautiously drop by drop down the side of the crucible, the lid being kept as far as possible over the top. The crucible was then placed in a slanting position on a pipeclay triangle and the edge of the lid heated with a small flame, till all free H_2SO_4 had been driven off; it was then placed upright and heated to a dull red heat; it was afterwards cooled, three drops of strong H_2SO_4 were added and a cautious heat was applied as before, except that this time the crucible was afterwards heated to a cherry red heat, for 10 mins. It was then cooled in a desiccator and weighed, heated again for 10–15 mins., cooled and again weighed. It was found that from 0·2 to 0·4 g. of calcium sulphate, which was the amount obtained from about 50 cc. of the solution, was the most satisfactory amount to deal with. Analyses were always made in duplicate, and estimations were repeated, if the results obtained differed by more than 1 mg. from one another. This gave an error of less than 0·5 %. In the majority of cases the difference was less than 0·5 mg.

For the phosphorus estimations the original solution was diluted 10 times and the 50 cc. taken for analysis were placed in a Kjeldahl distilling flask, 10 cc. of strong H_2SO_4 added, and boiled till fumes of H_2SO_4 appeared. The estimation was then carried on by Neumann's method [1902]. All results which did not fall within 0·1 cc. of one another in the titration against $N/2$ NaOH were repeated.

Experiments on Normal Rats.

Three experiments were carried out on normal rats to which the above basal diet was fed. At the time these experiments were performed, the poor biological quality of egg-white as a protein was not suspected [see Boas, 1924] and the loss of hair and accompanying decline in weight were attributed to an infection. However, although in two cases considerable loss of weight occurred during the last week of the experiment, it will be seen that this had little or no effect on the growth and calcification of the skeleton.

Exp. I. Four female rats of the same litter were put in a metabolism cage at the age of 27 days and kept there for a preliminary period of five days, followed by three periods of a week each, during which the retention of Ca and P was estimated. During the last week loss of hair, blepharitis and decline in weight appeared and the experiment was terminated abruptly as it was thought that the animals were infected with mange.

Exp. II. Four female rats all belonging to the same litter were observed during a preliminary period of one week followed by five consecutive periods of one week each, during which estimations of Ca and P retention were made. At the end of the first period the four animals were divided between two cages and the experiment continued thus. Slight signs of coat defect appeared

during the last week and the weight curve flattened but no loss of weight occurred. The amount of P fed to the rats as K_2HPO_4 was doubled during the fifth week.

Exp. III. Two female rats of the same litter were kept in metabolism cages for a preliminary period of one week followed by four periods of one week each, during which the Ca and P metabolism was studied. Loss of hair and loss of weight again occurred during the last week. One rat was killed and the long bones, kindly analysed by Dr Goldblatt, gave a Ca content equal to 11·22 % of the wet weight and 20·80 % of the dry weight; the average for normal rats of the same age in the investigations of Korenchevsky was 11·50 % wet weight, and 20·50 % dry weight. The other rat was put on a diet of bread and milk, cabbage and carrot, and in a short time completely recovered. In this experiment also the amount of K_2HPO_4 given was doubled during the last week.

The results of these experiments are shown in the accompanying table. As the amounts of uneaten food were not estimated, the actual amounts of Ca and P ingested cannot be given. From the amounts fed a rough estimate can be made. In the first week of each experiment it was of the order of 0·6 g. Ca and 0·4 g. of P per rat per week, and it rose by about 0·1 g. Ca and 0·08 g. P every succeeding week except where in Exps. II and III a larger rise has been already stated.

Retention of Ca and P in young female rats aged four weeks at the beginning of the first week. In Exp. I four rats were in one cage; in Exp. II four rats were in one cage during the first week and after that in two cages; in Exp. III only two rats were observed.

Exp. No.	Week of Exp.	Initial body wt (mean of 4 animals) g.	Final body wt (mean of 4 animals) g.	Ca retained per rat (average) g.			P retained per rat (average) g.			Ca/P retained
I	1	41	54			0·1519			0·1018	1·72
	2	54	73			0·1227			0·0575	2·02
	3	73	65			0·1254			0·0620	
					Totals	0·4000			0·2213	1·77
				Cage A	*Cage B*		*Cage A*	*Cage B*		
II	1	56	77			0·2182			0·1043	1·91
	2	77	100	Lost	0·2409	0·2409	Lost	0·1361	0·1361	
	3	100	115	0·2119	0·1914	0·2017	0·1452	0·1184	0·1318	1·55
	4	115	127	0·1439	0·1662	0·1551	0·1153	0·0828	0·0991	2·71
	5	127	135	0·1305	0·1429	0·1367	0·0803	0·0181	0·0492	
					Totals	0·9526			0·5205	1·83
		Mean of 2 animals	Mean of 2 animals							
III	1	45	70			0·2126			0·1754	1·24
	2	70	87			0·2000			0·1578	
	3	87	98			0·1643			0·0964	1·18
	4	98	94			0·0895			0·1184	
					Totals	0·6664			0·5480	1·22

The retention of Ca and P decreases week by week, and though by a mere consideration of the figures this decrease seems to be somewhat irregular this

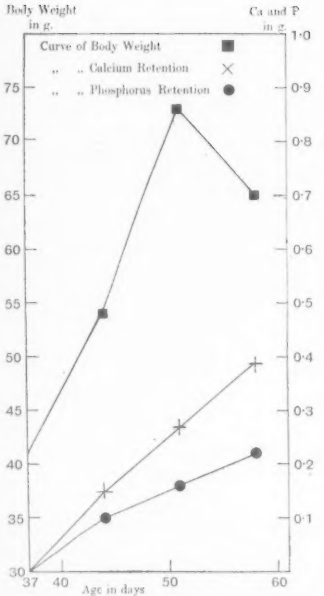

Fig. 1 (Exp. I). Curves of average increase in body weight and of average Ca and P retention per rat of the four female rats in Exp. I, plotted against the time in days.

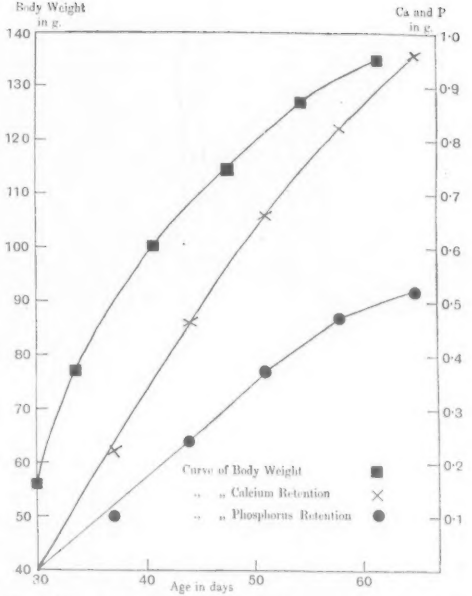

Fig. 2 (Exp. II). Curves of average increase in body weight and of average Ca and P retention per rat of the four female rats in Exp. II, plotted against the time in days.

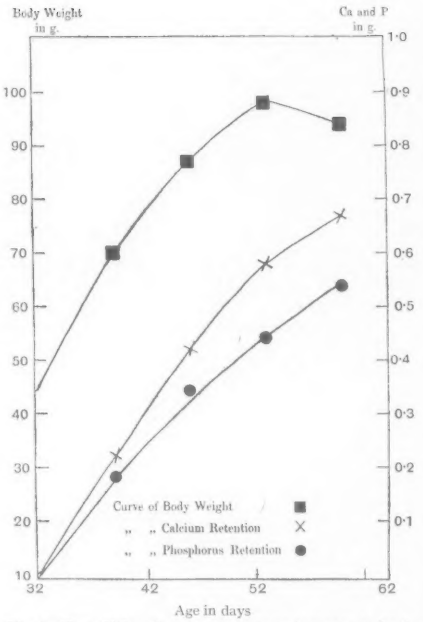

Fig. 3 (Exp. III). Curves of average increase in body weight and of average Ca and P retention per rat of the two female rats in Exp. III plotted against the time in days.

is due to the arbitrary periods into which the observation was divided. If x is taken as the unknown amount of Ca or P in the body of the rat at the beginning of the experiment and the successive increments are plotted against the time, a curve showing the weekly additions of Ca or P to the body is obtained. Figs. 1, 2 and 3 show the curves given by the above experiments together with the corresponding growth curves, and it will be seen that both Ca and P give smooth curves. The decline in weight at the end of the experiments, shown to be due to the inadequacy of egg-white as a source of nitrogen, does not seem to have affected the Ca retention.

The Ca : P ratio of the retention during successive periods in these experiments, which is given in the last column of the table, shows some variation. In the first two experiments it is higher during the last period than during the first, and in the third experiment it is approximately constant. This rise was shown in a more marked degree in the experiments described in the following paper [Boas and Chick, 1924] where the significance of it is fully discussed so that it need only be mentioned here.

SUMMARY.

A method for estimating the retention of Ca and P in young growing rats for periods of three to seven weeks is described.

Rats on normal diet showed a retention of Ca and P which declined week by week, and curves plotted from the figures obtained gave smooth curves similar in shape to the curve of normal growth.

In conclusion I wish to express my gratitude to Prof. C. J. Martin for suggesting this line of research to me, and for his invaluable help and advice throughout.

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LX. THE INFLUENCE OF DIET AND MANAGEMENT OF THE COW UPON THE DEPOSITION OF CALCIUM IN RATS RECEIVING A DAILY RATION OF THE MILK IN THEIR DIET.

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(*Received March 3rd, 1924.*)

Introduction.

There has been much difference of opinion as to whether cow's milk possesses any specific anti-rachitic properties and, if so, to what extent these may vary in degree according to the diet of the cow and the season of the year. The discovery of Mellanby [1919, 1921] showing the anti-rachitic value of certain animal fats for dogs and the correspondence between these values and their content of vitamin A [McCollum and co-workers, 1913, 1915, 1916] was generally interpreted to mean that vitamin A, the principle promoting growth and preventing xerophthalmia in rats, was also concerned with the calcification of bone and the prevention of rickets in the growing animal. Of the foodstuffs investigated, milk, butter and cream were among the richer sources of vitamin A, and that the content of this principle depends upon the diet of the cow has been abundantly substantiated. Kennedy and Dutcher [1922] showed that milk from cows receiving pasture food in summer was richer in vitamin A than milk from the same herd in winter upon a diet of dry fodder and hay, and that in winter the content of vitamin A could be further reduced by substituting timothy hay for alfalfa hay. Drummond, Coward and Watson [1921] demonstrated an increase in vitamin A in the butter prepared from the milk of cows when the herd was put out to grass in May, and a decrease later in the summer, in July, when the green food failed owing to drought, and oil-cake was re-introduced into the diet. These results have been confirmed by further investigations of Drummond and his colleagues [1924].

Recent work of McCollum and his co-workers [1922, 1, 2], however, points to the conclusion that the organic factor in diet concerned with deposition of calcium in the bones of growing animals is distinct from vitamin A, although in general the two food factors follow a similar distribution in nature and possess similar physical and chemical properties. A quantitative study of the resistance to heat oxidation of these two principles, as contained in cod-liver oil, has been made by Goldblatt and Zilva [1923], and the results are in support of this view. Certain investigators, for example Zucker and Barnett [1923], go further and maintain that the distribution of vitamin A and the anti-

rachitic organic factor is by no means so similar as has been supposed, that specific anti-rachitic properties cannot be demonstrated in milk or butter fat and that cod-liver oil, and probably also egg-yolk [see Hess, 1923], are substances apart, with a specific action upon calcium metabolism. A similar view, based on clinical grounds, has been expressed by Findlay [1922] and also by Galbraith [1923], and several observations are recorded in the older literature [Steinitz, 1903; Rothberg, 1907; Freund, 1909; Orgler, 1912] where addition of extra milk-fat to the diet of young infants actually lowered the retention of calcium in contradistinction to the effect of cod-liver oil. Many of these experiments were continued over too short a period after the change in diet was made, to yield very trustworthy results, and against the conclusions of these workers must be set the recent observations of Korenchevsky [1922], Korenchevsky and Carr [1923] and of Goldblatt and Soames [1923], who have shown that rats receiving a rickets-producing diet, can be protected by a butter or milk ration or by unheated caseinogen, although the amounts required are much greater than in the case of cod-liver oil.

Object of the present experiments.

The present experiments were made in connection with a research undertaken by Dr E. M. Luce in this Institute, the object of which was to study both the growth-promoting and anti-rachitic value of the milk given by the same cow under different conditions of diet and management. This research, of which a résumé has already appeared [1924], will shortly be published in detail. The criteria adopted by Dr Luce were growth, calcium content of the bones and prevention of rickets in young growing rats receiving the milk as an addition to a diet deficient in fat-soluble vitamins. Samples of the milk thus investigated were handed to us for parallel tests, in which the influence upon calcium and phosphorus retention was directly measured by metabolic experiments. The animals used were also rats which received small quantities of the milk as a supplement to a diet deficient in fat-soluble vitamins but adequate in other respects, including vitamins B and C.

Methods.

The method employed has been described in detail in the preceding paper by Boas [1924]. Each sample of the milk was tested by an experiment with two cages, each containing two young rats of the same sex, of about 40 g. weight at the beginning of the observation. The daily ration of milk was 5 cc. per rat, and was fed separately to each animal. The basal diet was the – A diet described by Boas, except that an attempt was made to correct the defects due to egg-albumin when employed as a sole source of protein. During the last two weeks of Exp. 1, fresh spinach was added to the diet in a ration of 1 g. daily per rat. McClendon and Shuck [1923] and also Goldblatt and Zilva [1923] were unable to demonstrate anti-rachitic properties in spinach, when administered to rats in amounts up to 5 g. daily.

In Exps. II and III, 10 % of the egg-albumin in the diet was replaced by heated, inactivated, caseinogen.

Each cage was provided with a control, containing two rats of the same litter and sex, and receiving a diet exactly similar to the above, except that the daily ration of fresh milk was replaced by 5 cc. of a reconstituted standard dried milk (prepared to contain 3·6 % fat, 3·3 % protein and 6·0 % carbohydrate) and in addition three drops (0·073 g.) of cod-liver oil daily were given to each rat. This provided a constant standard diet, complete in every respect, for comparison.

An account of the cow's diet and management has already been given by Luce, but is briefly recapitulated here for purposes of convenience.

Previous to the experiment the cow had been out at grass but was removed in July, 1922, and placed in a ploughed paddock upon a diet poor in fat-soluble vitamins, consisting daily of white maize meal, 3 lbs. 12 oz.; gluten meal, 3 lbs.; crushed oats and barley, 3 lbs.; mangolds, 50 lbs.; chaff, 18 lbs.; barley straw *ad libitum*. Four months later, on November 12th, the cow was placed in a completely dark stall without any change in diet until May 24th, 1923. A calf was born on January 27th. Milk sample XIV was taken during the last week of this period and was the pooled morning's milk of seven consecutive days.

Fresh green food, fresh meadow grass and clover, was then introduced into the cow's food, and by June 4th 1 cwt. per day was given and formed the entire diet, the animal remaining in the dark stall as before. Milk sample XVI was the pooled morning's milk of seven days at the end of this period which lasted for two months. On August 1st, 1923, the cow was put out to grass. The weather was very fine and sunny during the early part of August. Milk sample XVIII was taken in the middle of October, after eleven weeks of exclusive pasture feeding.

The milking was done with great care and extreme cleanliness, sterilised vessels being used. The milk was bottled, the air in the bottles displaced by carbon dioxide and the milk was then pasteurised at 70° for 20 minutes. The carbon dioxide was used to obviate risk of vitamin oxidation at this temperature. The milk was stored in a refrigerator room and was found to keep good for several months.

The three samples of milk tested had the following composition as regards content of calcium, phosphorus and fat.

Milk sample	Fat %	Ca %	P %
XIV	3·85	0·120	0·101
XVI	3·90	0·114	0·079
XVIII	5·10	0·122	0·082

Each observation lasted 5–7 weeks. During the first week the young rats were kept in the metabolism cages and were fed the appropriate diet, but no analyses were made. The observation proper began at the end of this preliminary period, when it was assumed that any disturbance caused by change of diet and environment would be over.

Table I. *Balance sheet for one week, showing Calcium and Phosphorus retained by two female rats aged eight weeks: mean weight at beginning of week, 61 g.; at end, 66 g. (Exp. III, Cage 1. 4th week.)*

Intake					Output		
Food offered	% Ca in the food	% P in the food	Ca in g.	P in g.		Ca in g.	P in g.
150 g. diet sample H	0·0309	0·1367	0·0464	0·2051	In urine, faeces and uneaten food	0·9226	0·9540
25 g. ,, J	0·0301	0·1331	0·0075	0·0333			
2·3996 g. $CaCO_3$	40	—	0·9598	—			
4·4003 g. K_2HPO_4	—	17·64	—	0·7761			
72·25 g. (70 cc.) milk sample XVI	0·1221	0·0831	0·0882	0·0600	Uneaten food, 39·42 g. (calc.)	0·2284	0·2290
		Total	1·1019	1·0745			
Uneaten diet 24·09 g. dry wt = 39·42 g. wet wt	0·0309	0·1367	0·2284	0·2290			
		Real Intake	0·8735	0·8455	Real Output	0·6942	0·7250
		Total retention			=Ca 0·1793	P 0·1205	
		,, per rat			=Ca 0·0896	P 0·0602	

In Table I is set out an example of the method used, showing the detail of the analyses made upon one of the four cages during the 4th week of Exp. III. The cage was Cage 1 and contained two female rats of mean weight 61 g. at the beginning, and 66 g. at the end, of the week, each receiving 5 cc. (5·1 g.) daily of Milk Sample XVIII.

Results.

Exp. I. Milk sample XIV. *Cow receiving dry fodder in a dark stall. (Table II and Fig.* 1.)

This observation, made with eight young rats, four of each sex and all from the same litter, lasted for six weeks. In Table II the calcium and phosphorus retention of the four rats, two male and two female, receiving milk XIV is averaged and compared with that of the four rats on the standard diet, with supplement of dried milk and cod-liver oil. The result is quite clear. In both cases the average amount of calcium and phosphorus retained per week diminishes with increasing age of the rat, but is consistently lower in case of the rats receiving milk XIV; in the 6th week of the experiment these rats actually show a negative balance. The total Ca retention per rat over the whole period was 34 % lower than that of the control rats, while the P retention was decreased by only 11 %.

In the curves in Fig. 1 the successive increments of Ca and P retained per rat per week, are plotted against time and illustrate how the discrepancy between the two sets of rats increases as they grow older. The curves of average increase in body weight in the two cases show no significant difference in growth between the animals of corresponding sex on the two diets, the growth in all cases being within normal limits.

There were no obvious differences post mortem, between the rats of the two series. In each animal the femora, tibiae, and fibulae were dissected.

the water content was determined and the proportion of calcium estimated by the method of Aron and Sebauer [1910]. The results showed that the control rats had slightly heavier bones, with more total Ca and a higher percentage but the differences are not perhaps very striking. The figures obtained for percentage of calcium, reckoned on dry weight of the bones (see Table IV) might be regarded as within normal limits in the experimental as well as in the control rats [see Korenchevsky, 1922].

Table II. *Calcium and Phosphorus retention in two male and two female rats on −A diet receiving 5 cc. daily of milk sample* XIV, *compared with two male and two female rats on − A diet with addition of 5 cc. standard reconstituted dried milk and three drops* (0·073 *g.*) *cod-liver oil daily.* (*All eight rats from the same litter.*)

	Four control rats on standard diet			Four rats receiving milk XIV		
Week of experiment	Mean body weight, g. (beginning of week)	Ca retained, g. per rat	P retained, g. per rat	Mean body weight, g. (beginning of week)	Ca retained, g. per rat	P retained, g. per rat
1	45	0·1877	0·1890	44	0·1376	0·1302
2	71	0·1533	0·1223	62	0·1024[1]	0·0784[1]
3	78	0·0633[1]	0·0712[1]	68	0·0825[1]	0·0711[1]
4	88	0·1396	0·1077	81	0·0852	0·1272
5	97	0·1075[1]	0·0790	86	0·0946[1]	0·0851
6	109	0·0835	0·0893	100	−0·0147	0·0940
End of 6th week	119	—	—	111	—	—
	Totals	0·7349	0·6585		0·4876	0·5860

[1] Figures calculated from results obtained with two rats only.

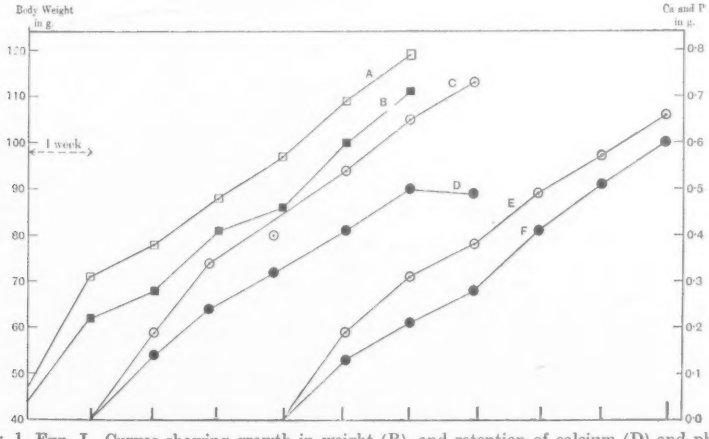

Fig. 1, Exp. I. Curves showing growth in weight (B), and retention of calcium (D) and phosphorus (F) in young rats on − A diet, receiving daily 5 cc. of milk sample XIV (cow on dry fodder in dark stall).
Curves A, C and E refer to growth in weight and retention of calcium and phosphorus respectively, of control rats from the same litter. Each curve represents average figures for four rats.

Exp. II. Milk sample XVI. *Cow receiving fresh green fodder in a dark stall.* (*Table III and Fig.* 2.)

The results of this experiment were similar to those of Exp. I. During the four weeks of the experiment the average total Ca retention of the female rats receiving milk XVI (Cage 1) was 0·3962 g. or nearly 20 % lower than that of the corresponding rats (Cage 3) on standard diet 0·4880 g. In case of the male rats (Cage 5), the retention was diminished by more than 40 % in comparison with the standard (Cage 7). The P retention was less affected, as was also the case in Exp. I, the decrease compared with corresponding control rats being 7 % in case of females and 19 % in case of males. The rate of growth in both series was almost identical.

In this experiment and in Exp. III, the uneaten diet was measured and the difference between this and the total food offered, appears in Tables III and V under the heading Intake. The figures show that an adequate amount

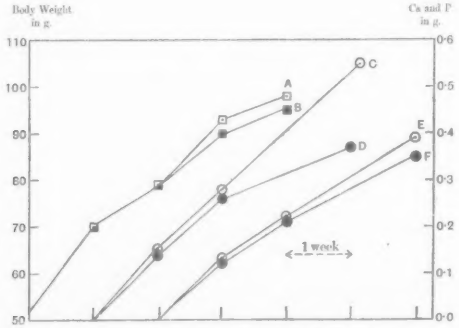

Fig. 2, Exp. II. Curves showing growth in weight (B) and retention of calcium (D) and phosphorus (F) in young rats on −A diet, receiving, daily, 5 cc. milk sample XVI (cow on fresh green food in dark stall).
Curves A, C and E refer to growth in weight and retention of calcium and phosphorus of control rats from the same litter. Each curve represents average figures for four rats.

of both Ca and P was ingested. The defect in the −A diet supplemented by milk XVI is therefore shown to be a failure to induce retention of calcium and, to a less degree, of phosphorus also. In the latter case the figures obtained are much nearer those of the control rats. The question of the ratio of Ca retained to P retained, presents points of some interest and is treated separately in a later section (p. 443).

The analysis of dissected bones, femora, tibiae and fibulae, at the end of experiment, showed a definite small diminution in Ca, for the experimental as compared with the control animals.

Fig. 2 shows curves of average total Ca and P retention in experimental and control animals as well as the average increase in body weight during the period of experiment.

Table III. *Calcium and Phosphorus retention in two male and two female rats on — A diet receiving 5 cc. daily of milk* XVI, *compared with that of two male and two female rats on — A diet with daily addition of 5 cc. standard reconstituted dried milk and three drops* (0·073 g.) *cod-liver oil. (All eight rats from the same litter.)*

	Four control rats on standard diet										Four rats receiving milk XVI									
	Cage 3 ♀					Cage 7 ♂					Cage 1 ♀					Cage 5 ♂				
		Ca, g. per rat		P, g. per rat			Ca, g. per rat		P, g. per rat			Ca, g. per rat		P, g. per rat			Ca, g. per rat		P, g. per rat	
Week of experiment	Mean body wt, g. Beginning of week	Intake	Retention	Intake	Retention	Mean body wt, g. Beginning of week	Intake	Retention	Intake	Retention	Mean body wt, g. Beginning of week	Intake	Retention	Intake	Retention	Mean body wt, g. Beginning of week	Intake	Retention	Intake	Retention
1	50	0·3992	0·1359	0·4187	0·1277	53	0·4220	0·1661	0·4444	0·1308	50	0·4330	0·1387	0·4499	0·1195	55	0·4291	0·1363	0·4530	0·1118
2	68	0·4441	0·1121	0·4443	0·0998	72	0·4224	0·1400	0·4213	0·0893	69	0·4800	0·1207	0·4727	0·0935	71	0·4519	0·1149	0·4472	0·0907
3	80	0·4307	0·1500	0·4159	0·1042	78	0·4086	0·1484	0·3952	0·1009	76	0·4865	0·1224	0·4245	0·1033	83	0·4950	0·1255	0·4738	0·0867
4	89	0·3681	0·0900	0·3865	0·0504	91	0·4325	0·1421	0·4555	0·0925	89	0·3775	0·0145	0·3883	0·0395	98	0·3955	−0·0400	0·4086	0·0442
End of 4th wk	90					100					93					104				
Totals			0·4880		0·3821			0·5966		0·4135			0·3963		0·3558			0·3367		0·3334

Table V. *Calcium and phosphorus retention in two male and two female rats on — A diet receiving 5 cc. daily milk sample* XVIII *compared with that of two male and two female rats receiving — A diet and in addition daily 5 cc. standard reconstituted dried milk and three drops* (0·073 g.) *cod-liver oil. (The four female rats and the four male rats were from two different litters.)*

	Four rats on standard diet										Four rats receiving milk XVIII									
	Cage 3 ♀					Cage 7 ♂					Cage 1 ♀					Cage 5 ♂				
		Ca, g. per rat		P, g. per rat			Ca, g. per rat		P, g. per rat			Ca, g. per rat		P, g. per rat			Ca, g. per rat		P, g. per rat	
Week of experiment	Mean body wt, g.	Intake	Retention	Intake	Retention	Mean body wt, g.	Intake	Retention	Intake	Retention	Mean body wt, g.	Intake	Retention	Intake	Retention	Mean body wt, g.	Intake	Retention	Intake	Retention
1	—	—	—	—	—	47	0·3960	0·1010	0·4068	0·0960	—	—	—	—	—	42	0·4143	0·1305	0·4185	0·1197
2	—	—	—	—	—	62	0·4876	0·1214	0·4798	0·1107	—	—	—	—	—	61	0·4327	0·0905	0·4159	0·0768
3	62	0·3965	0·0978	0·3951	0·0667	77	0·4511	0·1320	0·4481	0·0864	56	0·4091	0·0956	0·3952	0·0671	70	0·4482	0·1195	0·4378	0·0828
4	66	0·4354	0·0945	0·4326	0·0647	82	0·4879	0·1066	0·4867	0·0680	61	0·4368	0·0897	0·4230	0·0603	77	0·4952	0·1128	0·4813	0·0738
5	73	0·3978	0·0744	0·3974	0·0541	89	0·4845	0·1015	0·4319	0·0621	66	0·3983	0·0558	0·3865	0·0381	85	0·4687	0·0684	0·4579	0·0466
6	78	0·3856	0·0481	0·3820	0·0303	98	0·3915	0·0605	0·3879	0·0355	70	0·3479	0·0475	0·3338	0·0188	90	0·4099	0·0642	0·3968	0·0174
	81					99					72					92				
Total			0·3148		0·2158			0·6230		0·4587			0·2886		0·1843			0·5859		0·4171
			(Four weeks)					(Six weeks)					(Four weeks)					(Six weeks)		

The "Intake" given in Tables III and V is exclusive of 0·043 g. Ca and 0·030 g. P received weekly per rat in the milk ration.

Table IV. *Proportion of Calcium in bones (two femora, two tibiae and two fibulae) of the rats at the conclusion of Exps. I and II.*

Exp. No.	Cage No.	Sex	Nature of experiment	Rat No.	Wt of bone (fresh) g.	Dried at 110°	Ca found g.	% water in bone	% Ca: In fresh bone	% Ca: In dried bone	% Ca: In dried bone mean value
I	1	♀	Milk sample XIV	19	1·3082	0·7325	0·1416	44·01	10·82	19·32	19·66
			,,	21	1·3335	0·7346	0·1503	44·91	11·27	19·99	
	5	♂	,,	23	1·3641	0·7821	0·1524	42·67	11·18	19·48	19·29
			,,	25	1·3206	0·7450	0·1422	43·94	10·71	19·09	
I	3	♀	Control	20	1·5283	0·8966	0·1837	41·34	12·03	20·49	20·46
			,,	22	1·4057	0·8042	0·1642	42·81	11·68	20·42	
	7	♂	,,	24	1·5400	0·8684	0·2037	43·61	13·23	23·46	22·32
			,,	26	1·5984	0·8724	0·1847	45·41	11·56	21·17	
II	1	♀	Milk sample XVI	31	1·1062	0·5784	0·1093	47·71	9·88	18·90	19·08
			,,	33	1·2480	0·6785	0·1306	45·63	10·47	19·25	
	5	♂	,,	35	1·2126	0·6330	0·1085	47·80	8·95	17·14	17·82
			,,	37	1·4617	0·7783	0·1440	46·76	9·85	18·50	
II	3	♀	Control	30	1·1122	0·5938	0·1176	46·61	10·58	19·81	20·08
			,,	32	1·2700	0·6969	0·1418	44·10	11·16	20·34	
	7	♂	,,	34	1·2784	0·6993	0·1334	47·65	10·44	19·94	19·64
			,,	36	1·3128	0·7053	0·1364	46·27	10·39	19·34	

Exp. III. Milk sample XVIII. *Cow pasture-fed, August and September. (Table V and Fig.* 3.)

The result of this experiment forms a marked contrast with that of the two preceding ones, for, when the – A diet was supplemented with this sample of milk, it showed no significant inferiority to the same diet supplemented with cod-liver oil, as regards power of inducing calcium deposition.

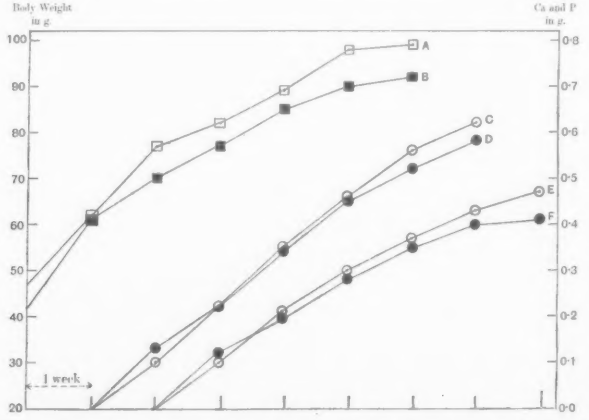

Fig. 3, Exp. III. Curves showing growth in weight (B), and retention of calcium (D) and phosphorus (F) in young rats, receiving daily 5 cc. milk sample XVIII (cow at pasture). Curves A, C and E refer to growth in weight, and retention of calcium and phosphorus respectively, of control rats from the same litter. Each curve represents average figures for two male rats.

Table VI. *Distribution of calcium and phosphorus in skeleton and soft tissues respectively, in seven rats at the end of Exp. III. Age of the rats,* 11 *weeks.*

No. of cage	Nature of experiment	Sex	Rat no.	Body weight, g. Gross	Body weight, g. Minus alimentary canal	Weight of skeleton, g. air dry	Weight of skeleton, g. dried at 110°	Weight of soft tissues (by difference) g.	Ca found, g. in soft tissues	Ca found, g. in skeleton	P found, g. in soft tissues	P found, g. in skeleton	Total Ca, g.	Total P, g.	Ratio Ca : P in whole body	Ratio Ca : P in skeleton	Ratio Ca : P in soft tissues	% in soft tissues Ca	% in soft tissues P	% in skeleton dried at 100° Ca	% in skeleton dried at 100° P	% in skeleton of total Ca	% in skeleton of total P
3	Control	♀	40	77·4	68·1	3·2143	2·8503	64·9	0·0423	0·7072	0·1463	0·3382	0·7495	0·4845	1·547	2·091	0·289	0·07	0·23	24·81	11·87	94·3	69·8
		♀	42	84·8	74·3	4·2175	3·2959	70·1	0·0581	0·8244	0·1628	0·3985	0·8825	0·5613	1·572	2·069	0·357	0·08	0·23	25·01	12·09	93·4	71·0
7	Control	♂	46	96·7	81·7	5·0089	3·7434	76·69	0·0495	0·8854	0·1609	0·4312	0·9349	0·5921	1·579	2·053	0·308	0·06	0·21	23·66	11·52	94·7	72·8
1	Milk XVIII	♀	39	70·9	59·4	3·6876	3·1037	55·7	0·0556	0·7420	0·1353	0·3611	0·7976	0·4964	1·607	2·055	0·411	0·10	0·24	23·90	11·63	93·0	72·7
		♀	41	73·0	62·4	3·9034	2·8023	58·5	0·0479	0·7002	0·1387	0·3430	0·7481	0·4817	1·554	2·042	0·345	0·08	0·24	24·99	12·24	93·6	71·2
5	Milk XVIII	♂	43	87·1	77·3	3·8597	3·2513	73·4	0·0550	0·7978	0·1619	0·3784	0·8528	0·5403	1·578	2·158	0·340	0·08	0·22	24·54	11·64	93·6	70·0
		♂	45	97·3	85·6	4·6548	3·6563	80·9	0·0989	0·8558	0·1875	0·4285	0·9547	0·6160	1·550	1·997	0·528	0·12	0·23	23·41	11·72	89·7	69·5
														Mean values	1·570	2·066	0·368					93·2	71·0

Table VII. *Showing ratio of calcium : phosphorus retained in the successive fortnightly periods of Exps. I, II and III.*

No. of experiment	No. of cage	Sex	Description	Period 1 1st and 2nd week ratio Ca/P	Period 2 3rd and 4th week ratio Ca/P	Period 3 5th and 6th week ratio Ca/P	No. of weeks	For whole period of experiment, ratio Ca/P
Group I:								
I	1 and 5	♂ and ♀	Receiving milk XIV	1·15	0·85	0·45	6	0·83
II	1	♀	,, ,, XVI	1·21	0·96	—	4	1·114
II	5	♂	,, ,, XVI	1·24	0·65	—	4	1·01
Group II:								
I	7	♂	Control on standard diet	1·14	1·06	1·27	6	1·15
II	3	♀	,, ,	1·09	1·55	—	4	1·28
II	7	♂	,, ,,	1·39	1·50	—	4	1·44
III	1	♀	Receiving milk XVIII	—	1·81	1·45	4	1·56
III	3	♀	Control on standard diet	—	1·46	1·45	6	1·46
III	5	♂	Receiving milk XVIII	1·13	1·48	2·07	4	1·41
III	7	♂	Control on standard diet	1·08	1·55	1·66	6	1·36

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p. 441, Table VII, *should read*

Group II:		Period 1	Period 2	Period 3
III	Receiving milk XVIII	—	1·45	1·81

This was also true as regards phosphorus retention in case of the male rats but in case of the female rats those receiving milk XVIII retained about 15 % less phosphorus than the controls, during the four weeks period of observation.

At the end of the experiment for each rat in the series an estimation was made of the Ca and P content of the whole animal, separate determinations being made for the soft parts and the skeleton. No significant difference between the two series was found, either in the amounts estimated or when these were reckoned per 100 g. body weight or in the ratio of calcium to phosphorus (see Table VI) in the skeleton and soft tissues respectively.

The separation of the skeleton was carried out in the way described by Donaldson [1919]. After removal of the alimentary canal the animal was weighed and the skin and organs were placed in a crucible together with the larger muscles. The carcase was then digested with about 200 cc. of a 2 % solution of Hudson's soap powder, the vessel containing the mixture being heated in a bath of boiling water 2–4 hours or until the bones could easily be separated with forceps—a lengthy business. The sample of soap powder used was free from P and contained only the slightest trace of Ca in the amount used.

After washing, the skeleton was dried in air, weighed, and then dried at 110° to determine water content. The dried bones were ashed in a crucible, the ash extracted with hydrochloric acid, and Ca and P determinations made in the ordinary way by McCrudden's and Neumann's methods. The dissolved portion of the digest, together with the washings of the skeleton, were evaporated to dryness and the residue added to the crucible containing the skin and other soft tissues and the whole was ashed together.

In order to see whether this method of separation affected the amounts or proportion of Ca and P found, results obtained by this method were compared with those got by analysis of the fresh dissected bone. This was done in the case of two corresponding rats, viz. No. 45 (male, cage 5) receiving milk XVIII, and No. 46 (male, cage 7) on standard diet. The bones selected for analysis were the femur and tibia. The results showed a consistent loss of about 5 % in the amounts of calcium and phosphorus found in the boiled bones. Part of this difference, at any rate, can be accounted for in the small amounts of ligaments and cartilage adhering to the dissected bones, from which the boiled bones were entirely free.

Calcium and phosphorus in femur and tibia analysed (i) *after dissection in the fresh condition,* (ii) *after boiling with* 2 % *soap powder solution.*

Rat No.	Method	Weight of bones dried at 110° g.	Calcium g.	Calcium %	Phosphorus g.	Phosphorus %	Ratio Ca/P
45 (milk XVIII)	Dissected fresh	0·3451	0·0667	18·47	0·0344	9·96	1·94
,,	Boiled	0·2907	0·0638	22·96	0·0316	10·84	2·02
46[1] (standard diet)	Dissected fresh	0·3379[1]	0·0668	19·76	0·0340	10·06	1·97
,,	Boiled	0·2720[1]	0·0636	23·38	0·0322	11·84	1·97

[1] Fibula also included.

Discussion.

The result of the metabolism experiments described above show clearly that young rats receiving a diet deficient in vitamin A (and in anti-rachitic properties) with a small daily addition of milk may show either a normal or defective retention of calcium and phosphorus according to the quality of the milk, this in turn depending on the diet and management of the cow.

When the cow was kept in a dark stall and received either dry fodder or fresh green food the milk was found to be defective in calcium-depositing properties, while under the influence of pasture-feeding, a combination of green food, sunshine and open air, she yielded a milk which given in a small daily ration induced normal calcium retention and calcification of the bones. We are inclined to attribute this difference in quality between the samples of milk to the effect of sunlight upon the cow. These results in the main confirm those obtained by Luce [1924] employing other methods. Her experiments showed in addition that the vitamin A (growth-promoting) value and the anti-rachitic value of the milk were similarly affected by changes in the treatment of the cow.

From the present series no information is obtainable as to the growth-promoting value of the sample of milk tested. The rats were not subjected to any preliminary period on −A diet without supplement, as in the methods adopted by Luce, and their reserves, together with the small daily ration of milk, sufficed to carry them over the six weeks experiment without any failure in growth. This was an essential feature in these experiments. If there had been a marked difference in the rate of growth of the animals receiving the samples of milk under investigation and of the control animals receiving cod-liver oil, differences found in the retention of calcium and phosphorus could have been attributed to this fact and need not necessarily have indicated a failure to deposit these substances. If, however, a striking difference occurred in the amounts of calcium retained with no significant difference in the rate of growth, then this difference must be due to faulty calcium metabolism.

This work is open to the criticism that a single metabolism experiment with each sample of milk provides too slender evidence for such generalisations as are made above. A long period of observation was found necessary in order to obtain trustworthy results and the labour involved in experiments lasting 5–7 weeks proved so great that it was found impracticable to duplicate them.

It might be thought that the higher content of fat in milk sample XVIII accounted for its superiority in inducing calcium deposition. The results obtained by Luce, however, showed that the proportion of fat in any sample bore no necessary relation either to the growth-promoting or anti-rachitic properties. One of the milks (sample VIII) investigated by her, of lowest value in these respects, had the highest percentage of fat found, 5·85 %.

The calcium and phosphorus metabolism of normal animals and the effect of deficiency of fat-soluble vitamins upon the ratio of calcium to phosphorus retained.

In the last column of Table VII is collected the ratio of total calcium to total phosphorus retained in Exps. I, II and III. In case of normal animals, that is the control animals in all three experiments and the animals receiving the pasture-fed milk in Exp. III, this figure lies between 1·23 and 1·56. These values are comparable with those observed previously in normal young rats

by one of us, and given in the preceding paper [Boas, 1924]: 1·24, 1·77, 1·85, mean value 1·65. In case of the rats receiving milk of poorer quality, however, in Exps. I and II, lower values were obtained: 0·85, 1·01, 1·11. That this difference is a real and significant one will become apparent when other facts have been considered.

Several workers have determined the ratio of total calcium to total phosphorus in the body of the young rat. Korenchevsky and Carr [1923] in a series of observations upon the constitution of the new-born rat, found the ratio of Ca to P equal to 1·00 in the whole fresh body minus the alimentary canal. McCann and Barnett [1922] made complete analyses of the calcium and phosphorus content of skeleton and soft tissues in case of both normal and rachitic rats. The mean value of the ratio of total Ca/total P for normal young rats, reckoned from their figures, varied between 1·38 and 1·65. In case of rachitic rats this ratio remained within normal limits when the growth of the rats had been limited, but in cases developing rickets with fair increase in body weight, the ratio was much reduced in value and varied between 1·06 and 1·33. From Buckner and Peter's [1922] figures the ratio total Ca/total P is 1·78 for male and 1·86 for female rats respectively at two weeks old. These results are at variance with those of other observers, being much too high in comparison with the figure of Korenchevsky and Carr and with those obtained by other workers for other young mammals. Bunge [1874] found this ratio to be 1·36 in a rabbit 14 days old, 1·47 in a dog 4 days old and 1·39 in a cat 19 days old. Abderhalden [1898, 1899] determined the ratio in the young rabbit and guinea-pig and found it to correspond with the ratio in which these elements were presented in the milk. Thus in the guinea-pig the Ca/P ratio was 1·25, in the milk 1·37; in the rabbit 1·36 and in rabbits' milk 1·46.

In the seven rats of Exp. III, which were completely analysed (see Table VI) and which are regarded as normal, the average Ca/P ratio for the whole body was 1·57. The age of these rats was 11 weeks and the average weight, minus the alimentary canal, was 73 g. After comparing this value with that obtained for the new born animal by Korenchevsky and Carr, it seemed of interest to find out whether this, presumably, gradual alteration in the relative content of calcium and phosphorus in the body, taking place in the early weeks of life was accompanied by a similar variation in the ratio of Ca/P retained, or whether this ratio remained constant during the successive weeks of the metabolism experiments. Table VII shows the ratio of Ca to P retained, calculated for successive fortnightly periods in Exps. I, II and III. It will be seen that the results of the observations fall into two groups. The first group concerns the rats fed with samples of milk of poor quality in Exps. I and II. The second group includes the observations upon the control animals in all three experiments and those on the rats receiving "pasture-fed milk" in Exp. III. In both groups the figure for the ratio Ca/P retained in the first two weeks, if one figure is excepted, is much the same and varies

between 1·08 and 1·24, but, whereas in the second group this ratio rises steadily as the animals grow older, in the first group it falls as consistently. This difference is what one would expect. Since the skeleton contains over 90 % of the total Ca of the body but only about 70 % of the total P, any failure in bone formation will naturally be more apparent in the amount of Ca retained than of P. As a result the value of the ratio Ca/P retained will fall more and more below the normal. The curves in Figs. 1 and 2 show that the defective retention of calcium is becoming more serious with increase of time, and that with increasing growth of the animal the skeleton is gradually becoming impoverished. It is noticeable that analysis of the bones does not at this stage show a very marked diminution in calcium content (see Table IV), although the figures obtained were consistently below those obtained in case of the control animals. The duration of the experiments, 5–7 weeks, was probably too short and shows rather the early stages of the gradual process of impoverishment. This observation confirms the conclusion arrived at by Schabad [1910] on the basis of his work with infants, that the early stages of rickets are to be detected only by a defective calcium balance in metabolism, and that changes in the skeleton, which can be diagnosed clinically, occur later.

The reason why the ratio of Ca/P in the animal's body should normally increase in case of young growing rats, at any rate up to the age of 11 weeks, is not very clear. The results obtained by Donaldson [1919] show that the growth of the skeleton is roughly proportional to the growth of the whole body during the early weeks of a rat's life. McClendon [1922] who noted an increase in the ratio Ca/P with increasing age, suggests that this is due to a relatively greater proportion of calcium carbonate being deposited in the bones as the animal grows older. According to Hoppe Seyler [1891], bone can be represented by a formula similar to that of apatite $Ca_{10}F_2(PO_4)_6$ in which the F_2 is replaced by CO_3. This formula $Ca_{10}CO_3(PO_4)_6$ has a Ca/P ratio of 2·152, whereas in $Ca_3(PO_4)_2$ the Ca/P ratio is 1·965. In case of the rats of 11 weeks old of which analyses are given in Table VI, the Ca/P ratio of the skeleton varied between 1·997 and 2·158, giving a mean value of 2·066. This figure agrees very well with the formula for bone suggested by Gabriel [1894], $Ca_3(PO_4)_2 + Ca_5HP_3O_{13}$; it lies between those calculated respectively for $Ca_3(PO_4)_2$ and for the "apatite" formula and suggests that in normal rats of this age the composition of the bone may be in a state of transition. The proportion of uncalcified material in the skeleton will normally tend to decrease as the animal grows up, and this fact will also tend to make the ratio Ca/P in the bones increase with increasing age.

Summary.

1. Experiments on calcium and phosphorus retention in young growing rats were used to investigate the power of various samples of milk to influence calcium deposition. The milk was fed to the rats in daily rations of 5 cc. and

was added to a basal diet arranged to be complete in all respects, but deficient in fat-soluble vitamins.

2. Three samples of milk were investigated, all obtained from the same cow, (1) after six months on a diet of dry fodder in a dark stall, (2) after two months on a diet of green food in a dark stall, and (3) after two months on pasture in summer.

3. Rats receiving samples (1) and (2) showed defective calcium retention as compared with control rats on the same basal diet supplemented with cod-liver oil and an equivalent ration of dried milk. The retention of phosphorus was also affected, but to a less degree. In these animals the ratio of calcium retained, to phosphorus retained, was found to decrease steadily during the early weeks of life whereas in normal animals this ratio shows a steady increase.

4. Rats receiving sample (3) showed no significant difference in calcium retention as compared with the control animals. Complete analyses of the bodies of these rats, aged 11 to 12 weeks, were made to show the distribution of calcium and phosphorus and the ratio of these substances to one another in the skeleton and soft tissues. The results obtained were practically identical for animals of both series.

5. The influence of cod-liver oil upon calcium deposition is not to be attributed to specific properties peculiar to that substance, but to its high content of fat-soluble vitamins, as a similar effect upon calcium deposition can be demonstrated in the case of cow's milk, the degree depending on the diet of the cow and the amount of sunlight to which she is exposed.

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LXI. PREPARATION OF COLLOIDAL GOLD FOR THE LANGE TEST.

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A PRELIMINARY communication of the writer's method was made to the *British Medical Journal* [1922], since when he has examined 250 cerebrospinal fluids with entirely satisfactory results. It is now proposed to redescribe the method, in part because its utility would appear to be established and partly because he has slightly modified it. Although Faraday and many authors since have considered neutrality a desideratum, the question of reaction has not been worked out and elaborate directions as to the preparation of the distilled water etc. are still given to the obscuring of the real issue. For what is happening when the reaction is adjusted at the end is in effect a neutralisation of the acids in the medium, whether present from the outset (as in the gold chloride and distilled water) or formed in the reduction. The mistake is frequently made of speaking of gold chloride as though only one preparation were known, whereas of course several are known; and they differ in acidity. Anyone can satisfy himself how true this is by carrying out the method about to be described, first using the double chloride of gold and sodium (the ordinary Scales brand used in photography) $AuCl_3$, NaCl, $2H_2O$; and then the hydrochloride ($AuCl_3$, HCl, 3 vel $4H_2O$).

Solutions prepared by Lange's modification of Zsigmondy's method, although looking well, often lack sensitivity and are acid; these may be called red slow samples.

Eskuchen using Eicke's method frequently made beautiful solutions which, for no known reason, would not react. In the absence of protective substances in the glassware (an important subject which will be dealt with later), the reason would seem to be that in these acid samples the particles are so small that the pathological constituents of the C.S.F. (or their experimental electrolytic equivalent) are unable to supply the energy necessary to aggregate them.

On the other hand, samples containing a frank bluish tinge (too alkaline and too sensitive) are liable to be produced by adding too much alkali; and curiously enough with certain reducing agents, *e.g.* glucose.

Between the beetroot red slow samples and the red blue fast samples there is to the eye a comparatively wide range of rose-coloured solutions which give satisfactory results. The main thing is not to read against some ideal colour in the mind's eye but against the red of the actual sample. The colour contrasts show well by reflexion in a pane of glass under which a piece of black paper has been placed on the bench.

The keynote of the method is simplicity. Ordinary laboratory distilled water is used, stale or fresh; any form of gold chloride (but the amount of adjuster will vary with the preparation); ordinary commercial formalin freshly diluted. The only novelty is the alkali added last—which may be called the adjuster—containing both the carbonate and the bicarbonate of potassium.

The reasons which led me to adopt a mixture of carbonates are these. I noticed that samples prepared with freshly made up K_2CO_3 were liable to be bluish red (corresponding already therefore to partial aggregation on the scale) and that the best samples were prepared with K_2CO_3 solution two or three weeks old; but if the solution were allowed to become yet staler, the samples tended to become rather pale like samples adjusted with $KHCO_3$ alone. This appeared to mean that at a certain point in the conversion of K_2CO_3 to $KHCO_3$ the optimum adjuster was formed. It was then attempted to determine the best proportions of the two carbonates and it was found after many trials that 2 % of each made an admirable adjuster which kept well.

The Method. Into a 250 cc. flask place 100 cc. ordinary laboratory distilled water. (We use an old tin-lined copper still.) Add 1 cc. of a 1 % solution of any preparation of gold chloride, say the double salt of gold and sodium (Scales brand) used in photography ($AuCl_3$, NaCl, $2H_2O$). Heat, and as the contents of the flask begin to bump, add 0·45 to 0·5 cc. of a 2½ % solution of formalin (freshly diluted). Remove flame to the edge of the wire gauze and add boldly, say from a Dreyer pipette, 18 drops (0·7 cc.) of the adjuster; then proceed cautiously drop by drop until faint red streaks appear. Take no notice of a *faint* blue tinge which sometimes appears before the red streaks. Add one more drop, when coarser streaks will come up, rapidly developing into a smart blush. Cease now.

The sample as it cools in the flask will assume a rich old rose colour to transmitted light, throwing on to white paper a red colour with a violet tinge. It will be dichroic and have a curious yellowishness to reflected light; 5 cc. of it will be completely precipitated by 1·5 cc. of 1 % saline in half-an-hour; it will give lilac luetic and paretic curves in appropriate cases and no greater reaction than 1 with known negatives; p_H 7·0–7·5.

The reagent should not be used quite fresh; we make our samples the evening before the test; on the other hand they may not keep, hence the advantage of preparing small quantities. Samples which keep well (no dichroism) are liable to be too stable for the test.

All apparatus used should be scrupulously clean to begin with and be used for nothing else but the test. Once, however, a set of apparatus has

been appropriated for the test, no more cleaning (of pipettes, for example) is desirable, except of the flask, which, if a film of metallic gold has deposited—as occasionally happens—may be cleaned out forthwith with a test-tube brush kept for the purpose, and distilled water. Curiously enough the best way to clean a new flask is to make a bad sample in it; this will get rid in a most efficient manner both of protective substances and electrolytes.

Conclusions.

(1) Colloidal gold possessing the required sensitivity for the Lange Test may be prepared in any laboratory in a few minutes with

(*a*) Ordinary laboratory distilled water.

(*b*) Commercial formalin.

(*c*) Any preparation of gold chloride; say the double chloride of gold and sodium used in photography (Scales brand). The fact is constantly overlooked that the various preparations of gold chloride differ in acidity, a consideration which renders an adjuster imperative.

(2) Neutrality is secured by adding an alkaline adjuster, consisting of potassium carbonate and bicarbonate, last, the reduction taking place at the same time.

(3) Some dichroism in the sample is not only unobjectionable but desirable.

(4) Care must be taken, as before, to see that the apparatus set aside for the test is free from protective substances and electrolytes.

My thanks are due to the Ministry of Pensions for permission to publish this communication.

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LXII. ON THE HYDROLYSIS OF THE ENDOSPERM OF *PHYTELEPHAS MACROCARPA* BY ITS OWN ENZYMES.

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Introductory.

Our knowledge of the enzymes which bring about the hydrolysis of the mannans is far less extensive than of those by which other polysaccharides are hydrolysed. There is no doubt, however, that they play an important role in the metabolism of certain plants, more especially during the germination process. Moreover since mannans are readily digested in the animal organism, it is more than likely that such digestion is the result of enzyme action. It would seem therefore that enzymes capable of hydrolysing mannans are widely disseminated in nature.

The first recorded observation of the hydrolysis of a mannan through the agency of an enzyme is that of Bourquelot and Herissey [1899] who showed that during the germination of the carob bean an enzyme is produced which converts the polysaccharides of the bean into mannose and galactose. In later communications these observers showed [1900] that enzymes (which they refer to as seminases), capable of hydrolysing mannogalactans and galactans into their derivative monosaccharides, occur in the fruits of fenugreek, lucerne, *Trifolium repens* and other plants. Subsequently they were found in orchid tubers [1901]. In some cases the enzyme action is comparatively slow, but it appears invariably to be accelerated by the presence of sodium fluoride. Grüss [1902] has observed the presence of similar enzymes in germinating date seeds.

Bierry and Giaja [1912] show that enzymes capable of hydrolysing mannans, galactans and celluloses are present in the alimentary canal of certain invertebrates, this being the first recorded instance of the presence of these enzymes in the animal kingdom.

Vegetable ivory (*Phytelephas macrocarpa*) is the usual source of mannose which is obtained by hydrolysing it with sulphuric acid. However, it will

be shown that the ivory nuts themselves contain an enzyme capable of bringing about the hydrolysis of the mannan, with the production of considerable quantities of mannose.

Pringsheim [1912] showed that if an infusion of certain bacteria be added to a suspension of ivory nut shavings the mannan is hydrolysed, giving mannose and a trisaccharide, mannotriose. This is presumably composed of three molecules of mannose and gives a characteristic osazone, forming stellate aggregates of needles, which are soluble in water and decompose at 196°.

Experimental.

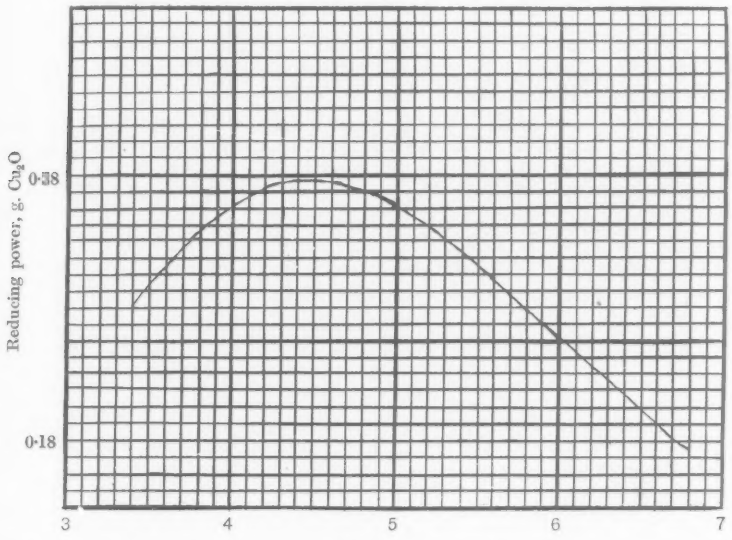
Whilst investigating the action of malt diastase on a suspension of ivory nut shavings it was observed that whereas diastase had no action if the suspension had been boiled, yet when added to the unboiled suspension, a considerable amount of reducing sugar was formed. This led to the discovery that the shavings contained an enzyme capable of hydrolysing the insoluble mannans and a series of experiments were carried out in order to determine the best conditions for this enzyme action.

Ten grams of the shavings were suspended in 200 cc. of water containing toluene, and the conversion was followed by withdrawing at definite intervals some of the solution, making up to a suitable volume and titrating with Fehling's solution. It was found that the maximum quantity of reducing sugar was formed when the temperature of incubation was 45°, whilst at a temperature of 60° the action appeared to be almost completely annulled. Further experiments indicated that the quantity of reducing sugars produced was approximately proportional to the concentration of the suspension, at least during the first 24 hours.

In order to determine the influence of hydrogen ion concentration on this enzyme action, a series of conversions were carried out in aqueous media at different hydrogen ion concentrations, the latter being varied by the addition of dilute sodium hydroxide or acetic acid to the initial liquid. The course of hydrolysis at 45° was followed, all other conditions being kept constant. It was found, however, that during hydrolysis there was an increase in acidity and that the suspensions attained a constant p_H value of 4·2 after two hours. This at once suggested the possibility that the shavings contained an appreciable amount of fatty matter, which was simultaneously hydrolysed by a lipoclastic enzyme also present. In one experiment an acidity corresponding to as much as 15 cc. $N/10$ NaOH was developed from 20 g. of the shavings. In order therefore to determine the concentration of hydrogen ions corresponding with the optimum activity of the enzyme, it was obviously necessary to extract the shavings with ether.

The fat-free shavings thus obtained were then used in a similar series of conversions at definite initial p_H values which in this case remained constant during the conversion. Four grams of the shavings were suspended in 100 cc.

of water and the conversions were carried out in exactly the same manner for two hours as in the previous series. The reducing powers of the different solutions after filtering were determined by titration with Fehling's solution and are shown in the diagram calculated as Cu_2O.



From the above diagram it will be seen that the p_H of optimum activity is about that produced by the formation of fatty acid during the conversion, viz. 4·2.

200 g. of shavings were allowed to hydrolyse in presence of toluene for six days in 1250 cc. of water at 45° when the solution was found to contain about 45 g. of reducing sugar, calculated as mannose. The liquid was pressed out, filtered, treated with lead acetate and the excess of lead removed by hydrogen sulphide.

After decolorising with norit it was concentrated *in vacuo* to a syrup. Its specific rotatory power was found to be $[\alpha]_D$ +13·3° which is just less than that of mannose $[\alpha]_D$+14·2.

When treated with phenylhydrazine in the cold a bulky precipitate of mannose hydrazone was formed, and after filtration and heating with phenylhydrazine acetate, two osazones were observed. One of these, which was soluble in water, gave the characteristics of Pringsheim's mannotriosazone but decomposed at about 170°. The other, which was insoluble in water, was shown to be glucosazone.

A portion of the sugar solution was used for the estimation of the trisaccharide present. It was found that after heating with dilute hydrochloric

acid for some time on a water-bath there was an increase in reducing power which corresponded to 7·5 % of mannotriose, assuming this to give three molecules of mannose on hydrolysis. Attempts were then made to isolate this polysaccharide but were not successful, the amount of osazone obtained being always very small. It was thought possible to effect a partial separation of the mannotriose by its sparing solubility in 92 % alcohol. Accordingly fractions were isolated, cryoscopic determinations of which gave values which although higher than the molecular weight of mannose were only indicative of a partial separation.

In these conversions it is highly probable that the trisaccharide is produced as an intermediate product which is finally hydrolysed to mannose. It was found that by allowing the enzyme action to continue until no further hydrolysis was observed (about 10 days) only mannose was produced. Attempts were then made to isolate mannotriose by stopping the reaction at an earlier stage. Accordingly a large scale conversion was carried out, the reaction being stopped by boiling after two days. The reducing power towards Fehling's solution before and after boiling with 1 % hydrogen chloride solution, appeared to indicate the presence of 10 % of trisaccharide. It was still however not possible to isolate this body.

It has not been found possible to obtain crystalline mannose direct from the solution after allowing the conversion to proceed under optimum conditions until the maximum reducing power was observed. Although syrups were obtained agreeing very closely with the optical constants of mannose they were not of sufficient degree of purity to crystallise from glacial acetic acid, possibly owing to the presence of galactose and laevulose produced by the hydrolysis of laevulo-mannan and galacto-mannan which have been shown to be present in the ivory nuts by Baker and Pope [1900].

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LXIII. THE ACCURATE ESTIMATION OF CALCIUM IN WHOLE BLOOD.

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The study of calcium in the blood, in relation to its metabolism in health and disease, of which very little is known, is based upon its accurate quantitative estimation.

In regard to methods, it is not considered necessary to recall the well-known work of Wright [1912], Vines [1921], Howland and Kramer [1920], Kramer and Tisdall [1921], and Laidlaw and Payne [1922] but reference may be made to the recent paper by Ling and Bushill [1922]. The method they adopt is based on the work of McCrudden [1909], Halverson and Bergeim [1917], and de Waard [1919]. The blood is incinerated in a platinum dish, the ash dissolved in concentrated hydrochloric acid and the solution washed into a centrifuge tube of special shape. In this tube the calcium is precipitated as oxalate by Halverson and Bergeim's modification of McCrudden's method. The calcium oxalate is washed twice, dissolved in dilute sulphuric acid and the solution titrated with $N/100$ potassium permanganate.

The calcium content of whole blood should be estimated. Although a number of investigators perform quantitative estimations of calcium on serum only, the data afforded is hardly sufficient for an accurate study of calcium metabolism. The corpuscular elements are known to contain calcium. Cowie and Colhoun [1919] found that red corpuscles contained calcium in somewhat smaller concentration than the serum.

The technique of the method I employ in the estimation of calcium in whole blood is both accurate and simple and is as follows.

About 8 cc. of blood are withdrawn from a vein and transferred either direct into a platinum dish, or first into a test-tube containing 0·1 g. of potassium oxalate and later into the platinum dish. The blood in the dish quickly loses weight by evaporation and should be weighed at once on a balance. The actual weight of the blood $\times \frac{1000}{1060}$ enables one to express the amount of blood in cc. sufficiently accurately. It is then dried on a boiling water-bath. When dry it is carefully incinerated over a low flame on an Argand burner, the temperature being gradually raised. Finally it is transferred to the more intense flame of a powerful Bunsen burner until incineration

is completed. It should be noted that calcium may be lost by spluttering or by "creeping over" the edge of the dish if the temperature is increased too quickly. Another method, which should be employed with care, is the addition of a little redistilled methylated spirit to the blood and the application of a light, repeat this three or four times and then incinerate. By this means blood can be completely ashed in about an hour.

The ash, which owing to the temperature of incineration has fused leaving a vitreous residue, consists chiefly of phosphate of iron. If this is treated with water and acetic acid only, the phosphate of iron does not dissolve and consequently a considerable proportion of the calcium is left behind. It is therefore necessary to treat the ash with hydrochloric acid which dissolves the vitreous residue completely. By evaporation to dryness on the water-bath, a granular residue is obtained from which the calcium is readily extracted with acetic acid, sodium acetate and water, leaving the iron phosphate in an insoluble form.

In practice, I take up the ash with about 5 cc. of water and five drops of strong HCl and after evaporation to dryness on the water-bath, the residue is treated with six drops of glacial acetic acid, 0·5 g. of sodium or ammonium acetate and about 8 cc. of water. A good excess of the acetate is required to ensure the removal of any free HCl which may be present or produced by chemical action. The liquid is reduced to about 4 cc. by evaporation on the water-bath, cooled and filtered through a filter paper (7 cm. in diameter) into a 10 cc. glass centrifuge tube of an ordinary water turbine pattern centrifuge. The lixiviation of the residue is repeated with another 2 cc. of liquid acidified with two drops of acetic acid and this passed through the filter.

To remove every vestige of calcium, it is necessary to wash the filter paper with at least another 15–20 cc. of water. But the whole procedure must be completed in the one centrifuge tube and there is only space available for another 3 cc. To overcome this difficulty, I remove the centrifuge tube, wash out the platinum dish with 4 cc. of water acidified with a few drops of glacial acetic acid, and pass this liquid through the filter paper into a glass evaporating dish. This is repeated three or four times, following which the liquid is evaporated on the water-bath to about 2 cc. and transferred to the filtrate in the centrifuge tube. The glass dish is then finally washed out with 1 cc. of water, which is added to the rest of the liquid in the centrifuge tube.

This procedure is essential, as I have on a number of occasions incinerated the paper after ordinary filtering and found that about 20–25 % of the total calcium had been retained in the filter paper. Six drops of ammonium oxalate solution are now added and the liquid heated. If the cloudy precipitate of calcium oxalate which appears is not satisfactory, add a few more crystals of sodium acetate to make certain of the complete replacement by acetic acid of the HCl which was first used. The precipitation of the calcium oxalate must be done in the presence of acetic acid, as in ammoniacal solution the oxalates of both calcium and magnesium are precipitated.

The next step is to render the precipitate of calcium oxalate sufficiently granular to enable it to be readily centrifuged. I tried adding six drops of a saturated solution of an electrolyte, either aluminium acetate or sodium chloride, and heating the centrifuge tube over a boiling water-bath for 20 minutes. This acted perfectly when estimating solutions containing known quantities of calcium, but on applying the principle to blood estimations, I found a considerable amount of the calcium retained in the supernatant fluid pipetted off after the first centrifuging. The same thing happened, when, following the method recommended in ordinary quantitative estimations of calcium in solutions, I made the solution alkaline with ammonium hydroxide and let it stand for 20 minutes with the intention of subsequently rendering it acid with acetic acid to redissolve the magnesium oxalate. Thus it is necessary, after adding the oxalate, to heat the tube over a water-bath for at least three hours to insure the precipitation of the total amount of calcium present. The oxalate is then washed three times by centrifuging and removing the supernatant fluid. The latter is done by means of a glass pipette with an upturned point to prevent sucking up any of the precipitate. I do not recommend decanting off the supernatant fluid. Test the last wash in a small test-tube, by adding a drop each of NH_4OH and $CaCl_2$ and bring to the boil. If cloudiness appears add a few drops of glacial acetic acid which should make the solution perfectly clear. If cloudiness persists, there is still soluble oxalate present and the calcium precipitate must be rewashed and the washings retested. Excess of soluble oxalate must be removed, otherwise it will react with the permanganate and give an artificially high calcium value.

Dissolve the washed calcium oxalate in 3 cc. of distilled water containing four drops of dilute H_2SO_4 (1 in 10), heat to 60° and titrate with $N/100$ potassium permanganate solution. The end point is the faintest pink and the solution should again be heated to 60° when this appears—the addition of the permanganate solution having reduced the temperature to below that figure. The thermometer should be used for stirring the solution and washing off any calcium which may adhere to the upper part of the tube during the centrifuging process. It is important as a control to titrate four drops of the H_2SO_4 in distilled water with permanganate to ascertain whether there is any organic matter present in these reagents. The recognition of the pink end point is dependent upon the personal factor in the estimation and is thus liable to error. To insure accuracy I have made use of the reaction of acid permanganate solution and KI and the subsequent titration of the iodine liberated with thiosulphate, using starch as indicator. This reduces the error to a minimum if the $N/100$ solutions employed are accurate. After obtaining the end point which should persist for at least a minute, run in an excess of $KMnO_4$ (1 cc.) from the burette. Transfer to a beaker and wash the outside of the mouth as well as the inside of the tube with distilled water, to make certain that all the solution has passed to the beaker. Add two large crystals of a sample of potassium iodide which has been tested and found to

contain no free iodine—there must be an excess of KI—and titrate the yellow coloured iodine solution which results with $N/100$ sodium thiosulphate, using starch as indicator. The end point disappearance of the blue coloured compound of starch and iodine is one of the most delicate indicators known. Subtract the amount of sodium thiosulphate used from the total potassium permanganate figure and you have the actual end point. This should coincide with the first reading of the burette and represents the exact amount of $KMnO_4$ required to oxidise the oxalic acid in the centrifuge tube after the decomposition of the calcium oxalate precipitate by sulphuric acid.

The burettes (10 cc.), the pipettes and standard flasks used for making up $N/100$ solutions, should be of the best Charlottenburg or National Physical Laboratory standard pattern and should be carefully cleaned and dried after use. The $N/10$ solutions must be made up with the best analytical reagents, weighed out on an accurate balance by the investigator himself, and the solutions freshly prepared at frequent intervals. The $N/10$ potassium permanganate should be tested every fortnight against an accurately prepared stable solution such as $N/10$ oxalic acid, and the thiosulphate against the $KMnO_4$ solution. The thiosulphate solution is particularly liable to deterioration and must be kept in a dark bottle in a dark place. This applies to the $KMnO_4$ solution as well. The $N/100$ solutions, prepared from $N/10$ solutions, should be made up during the last washing of the calcium oxalate and used immediately. All reagents used must be free from calcium. The more concentrated the solutions are, the more liability there is to contamination by calcium salts.

The accuracy of the method is illustrated below:

Case 1. 20 cc. of blood were taken from a student's arm and divided into two parts.

Part I. Weight of blood = 8·84 g.

Volume ,, $= 8{\cdot}84 \times \frac{1000}{1060} = 8{\cdot}3$ cc.

Titration figure = 2·25 (cc. of $KMnO_4$) $\times$ 0·2[1] = ·45.

$$\cdot 45 \times \frac{100}{8{\cdot}3} = 5{\cdot}42 \text{ mg. of Ca per 100 cc. of blood.}$$

Part II. Weight of blood = 11·699 g.

Volume ,, = 11·03 cc.

To the blood, before incineration, were added 2 cc. of an accurate solution of calcium chloride containing 0·2 mg. Ca in each cc.

Titration figure = 5·05 (cc. of $KMnO_4$) $\times$ ·2 = 1·01 mg. Ca.

1·01 − 0·4 mg. Ca added = ·61 mg. Ca present in the blood.

$$\cdot 61 \times \frac{100}{11{\cdot}03} = 5{\cdot}53 \text{ mg. Ca per 100 cc. of blood.}$$

There is thus a difference of only ·11 mg. Ca per 100 cc. of blood between the two estimations, which is well within the limits of experimental error.

[1] 1 cc. of $N/100$ solution of a calcium salt contains 0·2 mg. Ca.

Further results obtained in a similar manner are as follows:

	Volume of blood in cc.	Titration figure cc. of $N/100$ $KMnO_4$	Mg. of Ca per 100 cc. of blood
Case 2	11·0	3·0	5·45
,, 3	7·8	2·3	5·90
,, 4	10·5	3·0	5·71
,, 5	8·49	2·4	5·65
,, 6	10·94	3·15	5·76
,, 7	8·72	2·8	6·42
,, 8	11·20	3·85	6·87

The blood from Case 6 was incinerated in about an hour by the addition of methylated spirit in the manner described above. All the investigations were done on normal individuals. The findings range from 5·42 to 6·87, the average being 5·8 mg. Ca per 100 cc. of blood. It should be noted that the results are given in milligrams of calcium and not lime.

Kramer and Tisdall [1921] found that the calcium content of whole blood in normal persons varied from 5·3 to 6·8. An average of 5·8 mg. Ca per 100 cc.

All these figures vary within comparatively wide limits and in using calcium estimations for clinical purposes, it is obviously futile to attempt to draw conclusions from very slight variations. It is possible that in some cases sufficient attention has not been given to technique and consequently I have thought it worth while to set forth step by step a technique which, if followed exactly, should give accurate results.

Cowie and Colhoun [1919] state "that the limits of variation of blood calcium in normal individuals are marked. Lyman [1917] found the same variations in normal individuals which he was unable to explain. Determinations of the number of corpuscles and of the specific gravity of the blood might aid in clearing up this point by giving an idea of the concentration." Their figures range from 6·64 to 9·08 with an average of 8·45 mg. of Ca per 100 cc. of blood.

Conclusions.

(1) The titration of calcium oxalate by $N/100$ potassium permanganate is an extremely accurate and comparatively simple method of estimating calcium in the blood, if due attention is paid to technique.

(2) Using the method described above I found that normal values in human blood range from 5·42 to 6·87 with an average value of 5·8 mg. of Ca per 100 cc.

Finally I wish to thank Dr G. Roche Lynch and Mr John Webster for the advice and assistance they have very kindly given me.

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LXIV. NESSLERISATION AND AVOIDANCE OF TURBIDITY IN NESSLERISED SOLUTIONS.

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One of the main conclusions reached by Stanford [1923] in a recent paper, under the title given above, is stated as follows: "No condition could be discovered in which clear solutions could be obtained by 'direct Nesslerisation,' as suggested by Folin." In a preceding part of the paper, Stanford further writes as follows:

In the past few years Folin has described several rapid methods for the estimation of nitrogen or urea nitrogen in products of animal origin "by direct nesslerisation," that is to say, the reaction product is treated with Nessler's solution and compared with a nesslerised standard. Cole [1920] has stated that he was unable to repeat these experiments because of the turbidity of the resulting solutions. The trouble seems also to have occurred to Folin himself, for he says in a later paper, "if the solution be turbid, centrifugalise a portion of it." A strange counsel, seeing that the turbidity is due to minute oil drops of the dark brown colouring matter which it is proposed to estimate.

The direct nesslerisation methods have been taught to hundreds of students and are in daily use in scores of American hospital laboratories. The avoidance of turbidity was necessarily the chief difficulty that had to be overcome in the development of the process and it is rather naïve on the part of Stanford to assume that I "in a later paper" advised the removal of the coloured turbidity by means of the centrifuge. The centrifuging is recommended in connection with total nitrogen determinations to remove the silica often produced by the boiling mixture of phosphoric and sulphuric acids during the preliminary digestion. On page 133 of my laboratory manual [1922] occurs the following:

If the unknown nesslerised digestion mixture is turbid, centrifuge a portion, giving a crystal clear fluid above a white sediment (silica). If the sediment is coloured, the nesslerisation was not successful and the determination must be discarded.

On page 237, under directions for determining the non-protein nitrogen in blood, the following helpful hints are given:

Many seem to have trouble in obtaining perfectly clear solutions when nesslerising the digestion mixtures obtained with blood filtrates. The cause is lack of suitable alkalinity in the Nessler solution. The following data will help to overcome the difficulty.

Twenty cubic centimetres of normal hydrochloric acid may be titrated with the Nessler solution and if the solution is substantially correct, a good end point will be obtained at 11 to 11·5 cc. with phenolphthalein as indicator.

If an end point is obtained much below 11 cc., as at 9·5 cc., the Nessler solution is too alkaline and turbidity is likely to occur.

Turbidity due to excess of alkalinity may likewise be produced because the sulphuric-phosphoric acid mixture is too weak. If 5 cc. of the dilute acid (1 : 1) are further diluted 10 times (to 50 cc.), 10 cc. of the solution so obtained, when titrated with the Nessler solution and phenolphthalein as indicator, should give a fairly good end point at 9 cc. to 9·3 cc.

Again on page 285, under directions for preparing the Nessler reagent, is given the following:

In the absence of modifying circumstances, such as the presence of much acid or alkali this (Nessler) reagent should be added in the proportion of 10 cc. per 100 cc. of the volume to which the nesslerised solution is to be diluted. As a general rule the volumetric flask (or volumetric test tube) should be at least two-thirds full before adding the Nessler reagent. If attention is not given to this detail, turbid mixtures are obtained, and turbid solutions must never be used for colour comparisons.

When Stanford concludes that direct nesslerisation is unattainable, and that clear solutions can be obtained only by adding the Nessler solution "*drop by drop with constant shaking*," this proves only that he is using an unsuitable reagent. Under my directions for determining the ammonia in urine, page 129, are given the following directions for nesslerising 0·5 mg. of ammonia in a 100 cc. volumetric flask:

Transfer 10 cc. of Nessler's solution (see page 283) to a measuring cylinder. Now give the volumetric flask a vigorous whirl so as to set the solution spinning within the flask and add at once the whole of the Nessler solution in the cylinder....If the process of nesslerisation has been successful, a deep red but crystal clear solution is obtained. If it is not perfectly clear, throw it away and prepare a fresh standard. With a little experience no trouble is encountered in getting clear solutions.

The preparation of the Nessler reagent here referred to is described in the paper entitled: "A System of Blood Analysis" [Folin and Wu, 1919]. It is not the best reagent for each individual purpose, but has been so modified as to be fairly satisfactory for all the different nesslerisations involved in blood and urine analysis. It is least satisfactory for nesslerising the urea of urine after decomposing with Jack bean extracts.

The most disturbing impurity is magnesium in any form, and it is because of its magnesium content that tap water cannot be used for the preparation of solutions which are to be nesslerised[1].

[1] In the course of the past dozen years, I have repeatedly returned to the subject of colorimetric nitrogen determinations and have made various observations as to the applicability and limitations of Nessler's reagent. For the convenience of any one wishing to make further studies on this topic I venture to give the following specific references to the *Journal of Biological Chemistry*, vol. **11**, pp. 493, 496; vol. **26**, pp. 474–5, 478–80, 483, 497–98; vol. **38**, pp. 111–12. Stanford evidently has not had access to this literature.

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